The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 12, December 25, 1958

Vol. 45, No. 12 **December, 1958**

THE JOURNAL
OF
BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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The Journal of Biochemistry was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this Journal has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

ON THE EFFECT OF BILE ACIDS ON CARBOHYDRATE METABOLISM

I. HYPERGLYCEMIC ACTION OF SODIUM CHOLATE AND DESOXYCHOLATE

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(Received for publication, January 8, 5958)

A number of workers observed hypoglycemia following subcutaneous or oral administration of bile acids to animals (*1–4*) and human beings (*5, 6*) in quantities of several mg. per kg. body weight under either fasting or well fed condition. Morever, it was reported that the acids given to diabetic patients caused a decline of blood sugar level and a suppression of urinary excretion of glucose (*5, 7*). But the effect was not appreciable in the case of diabetes with severely damaged Langerhans' islets (*5*) and in depancreatized dogs (*3*). From these observations it has been believed that bile acids stimulate the production of insulin.

The author undertook to elucidate the mechanism of the hypoglycemic action of cholic acid and other bile acids by administration of larger amounts of the acids to rabbits subcutaneously, intravenously and in a few instances rectally. Contrary to the expectation, a hyperglycemic response, instead of hypoglycemic one, was observed when the dose of the acids was increased beyond a certain amount. The experimental findings will be briefly reported in this paper, but it is restricted to the cases observed in a short time after the administration, with the exception of three cases.

Furthermore, still larger doses, 600 to 800 mg. per kg. body weight, gave rise to chronic hyperglyceima and the rabbits fell into diabetic conditian, as will be reported in the next paper.

EXPERIMENTAL

Animals—Male adult albino rabbits weighing 2.0 to 2.5 kg. were fasted for 24 to 30 hours before use.

Analyses—Blood sugar was determined by Hagedorn-Jensen's method and Roe's anthrone method (*8*), and reduced glutathione in blood by Fujita and Iwatake's method (*9*).

Sodium cholate and other bile acid salts were purchased from Minophagen K. K.

RESULTS

The blood sugar curves following subcutaneous injection of sodium cholate in various doses are illustrated in Fig. 1. In confirmation of the earlier workers the dose of 6 mg. per kg. body weight caused a decrease of blood sugar as shown in Curve A. But when 43 mg. per kg. body weight of sodium chalate were injected hypoglycemia was not observed (Curves B and C) and with an increased dose of 87 mg./kg. the level of blood sugar showed a slight elevation. Similar results were obtained by administration of sodium desoxycholate as seen in Fig. 2. On the contrary, no definite hyperglycemic effect was seen when taurocholate, glycocholate or dehydrocholate was administered (Fig. 3).

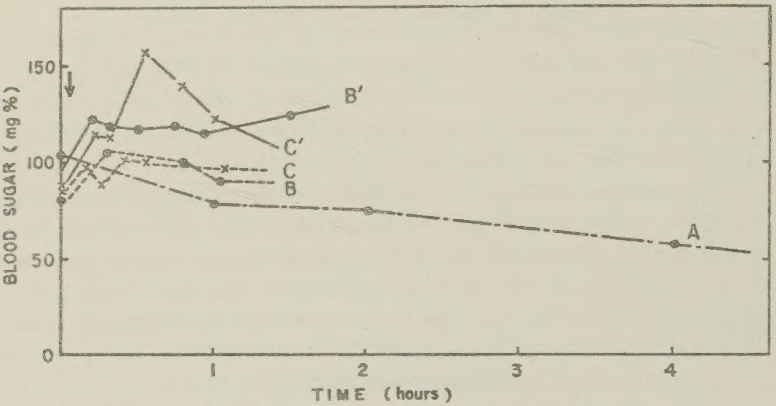

FIG. 1. Blood sugar curves following subcutaneous injection of various doses of sodium cholate in 1 per cent solution.

A, 6 mg./kg., Rabbit No. 7. B, 43 mg./kg., Rabbit No. 1.
C, 43 mg./kg., Rabbit No. 2. B′, 87 mg./kg., Rabbit No. 1.
C′, 87 mg./kg., Rabbit No. 2.

Experiments using the same animals were done with intervals of more than one week.

The hyperglycemic effect of cholate and desoxycholate was also observed in rectal administration. Again, glycocholate revealed no effect on blood sugar level (Fig. 4).

When the dose of sodium cholate was increased further to 300 mg. per kg. body weight or more the animals previously fasted developed a marked hyperglycemia which lasted for over 48 hours in one case (Rabbit No. 18) as illustrated in Fig. 5. In some experiments the animals were not fastad prior to the injection of cholate but fed on the usual stock diet. In these cases no appreciable hyperglycemic response occusred, even though large amounts of cholate were administered. For example, the Rabbit No. 18 which had been fed ordinarily showed only slight elevation of blood sugar level after injection of cholate (Curve V in Fig. 5). After about one

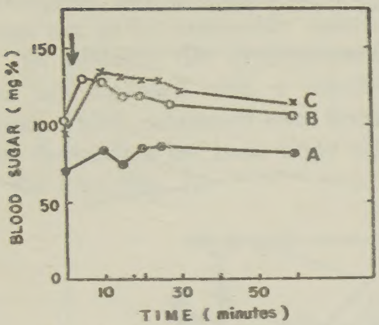

FIG. 2. Blood sugar curves following intravenous injection of various doses of sodium desoxycholate in 1 per cent solution.

A, 10 mg./kg., Rabbit No. 2.
B, 30 mg./kg., Rabbit No. 7.
C, 50 mg./kg., Rabbit No. 3.

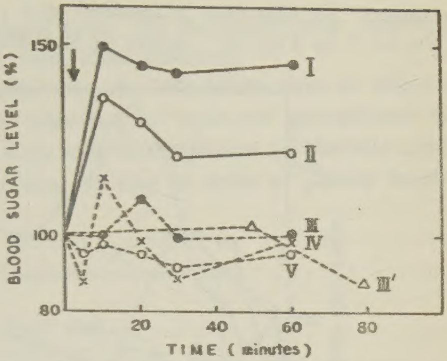

FIG. 3. Effect of various kinds of bile acids given intravenously on blood sugar level.

I, 50 mg./kg. sodium desoxycholate (1 per cent), Rabbit No. 3.

II, 86 mg./kg. sodium cholate (1 per cent), Rabbit No. 5.

III, 50 mg./kg. sodium taurocholate (1 per cent), Rabbit No. 6.

III', 375 mg./kg. sodium taurocholate (5 per cent), Rabbit No. 26.

IV, 65 mg./kg. sodium glycocholate, (1 per cent), Rabbit No. 5.

V, 52 mg./kg. sodium dehydrocholate (1 per cent), Rabbid No. 5.

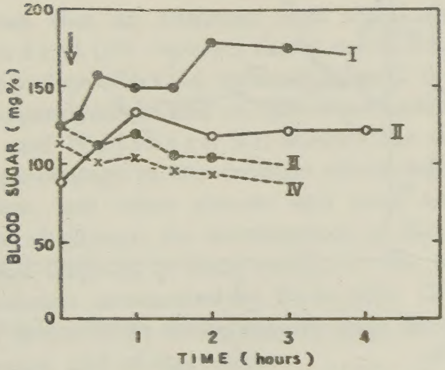

FIG. 4. Hyperglycemia induced by rectal administration of various kinds of bile acids in 1 per cent solution.

I, 65 mg./kg. sodium desoxycholate, Rabbit No. 5.
II, 200 mg./kg. sodium cholate, Rabbit No. 86.
III, 200 mg./kg. sodium glycocholate, Rabbit No. 5.
IV, Control, 40 ml. of physiological saline, Rabbit No. 5.

week the same animal was fasted for 40 hours and then injected with sodium cholate. In this case a marked and prolonged hyperglycemia was induced (Curve I in Fig. 5). Some of the animals died within a few hours after the intravenous injection of cholate. As shown in Fig. 6, a rabbit (No. 10, Curve I) given 300 mg./kg. cholate developed an appreciable hyperglycemia but two rabbits (No. 14, Curve II and No. 17, III) given

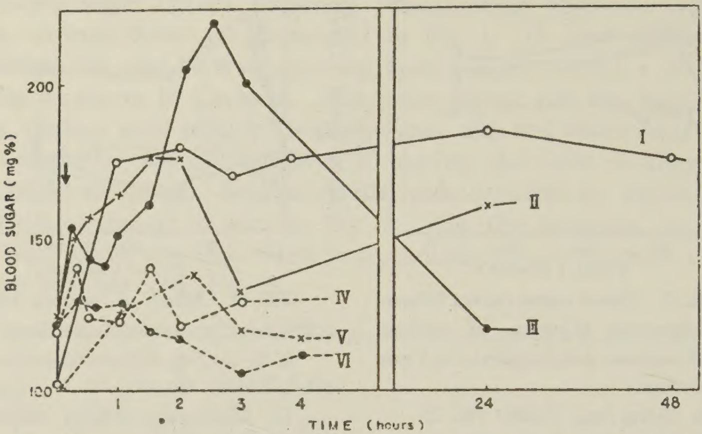

FIG. 5. Prolonged hyperglycemia induced by intravenous injection of large doses of sodium cholate in 5 per cent solution.

I, II and III, 300 mg./kg. of sodium cholate were given to the Rabbits No. 18, 13 and 11 which had been fasted for about 40 hours. IV and V, 300 mg./kg. of sodium cholate were given to the Rabbit No. 17 and 18 without previous fasting. VI, the same experiment with 400 mg./kg. cholate, Rabbit No. 16.

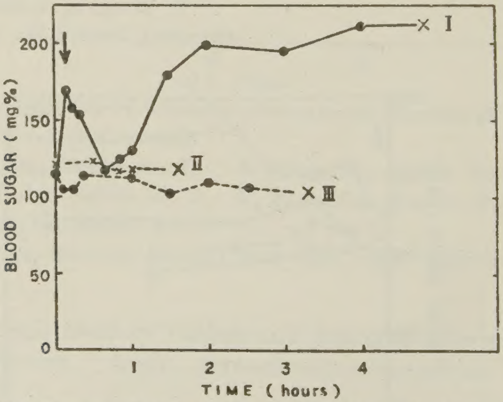

FIG. 6. Fatal cases after intravenous injection of sodium cholate. in 5 percent solution.

I, 300 mg./kg., Rabbit No. 10. II, 200 mg. of sodium cholate and 40 mg. of sodium desoxycholate per kg., Rabbit No. 14. III, 200 mg./kg., Rabbit No. 17.

The mark × indicates the death of the animal.

200 mg./kg. cholate did not. From these facts, the hyperglycemic response seems not to be necessarily associated with fatal outcome.

In addition, the hyperglycemia following the administration of cholate was accompanied with a significant decrease of reduced glutathione in blood, which continued over several days after the injection of the bile acid, and the rabbits lost their body weight day by day in spite of plenty intake of the diet (Fig. 7).

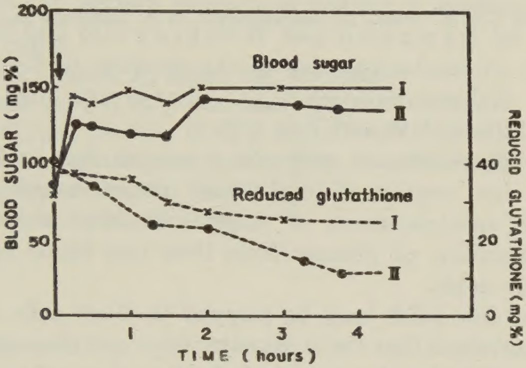

FIG. 7. Increase of sugar and decrease of reduced glutathione in blood following administration of sodium cholate.
I, 300 mg./kg., Rabbit No. 26. II, 350mg./kg., Rabbit No. 27.

DISCUSSION

It is well known that an adequate dose of alloxan induces diabetic condition, but Kendall (*10*) reported that a minor dose of less than 50 mg. per kg. body weight had no effect, *i.e.* no fluctuation of blood sugar in initial stage, no chronic hyperglycemia and no hyperlipemia following the injection of alloxan. Kass and Waisbreu (*11*) showed that alloxan diabetes was induced in higher percentage in the animals which had been fasted before use than in fed ones, and when glucose had been administered 6 hours before the injection of alloxan the development of diabetes was prevented. They attributed these findings, at least partly, to the lowered secretion of insulin in fasted animals as revealed by Best (*12*). The results presented here by the author seems to be in agreement with the foregoing works in the influence of dosage and of starvation.

Recently, Ashmor and Hastings, as transmitted by Knox (*13*), observed that glucose-6-phosphatase was activated definitely by bile acids. This finding is very interesting because both bile acids and glucose-6-phosphatase are produced and contained only in liver cells (*14*). Cori and Cori (*15*) revealed that glucose-6-phosphatase was almost completely absent in glycogen storage disease (von Gierke's disease) which was distinguished in high glycogen content in liver, heart, kidney and other organs, hypoglycemia in fasting state and lack of hyperglycemic response to adrenalin. In contrast to glucose-6-phosphatase which hydrolyses the substrate in the physiological pH range (optimum pH=6.8), unspecific acid phosphatase and alkali phosphatase have practicolly no action on glucosephosphate at the physiological acidity. The activity of phosphoglucomutase, however, was not impaired in the glycogen storage disease. It seems that glucose-6-phosphatase liberates glucose into the circulating blood from glucose-6-phosphate of liver cells, thus controling the blood sugar level.

Starvation produces a marked increase of glucose-6-phosphatase in the liver as reported by Langdon and Weakley (*16*) and by Weber and Cantero (*14*). A similar increase of the enzyme in liver was observed also in alloxan diabetes by Ashmor, Hastings and Nesbett (*17*) and by Langdon and Weakley (*16*).

From these observations it might be presumed that under the condition of animal with low content of insulin and increased activity of glucose-6-phosphatase the administration of large quantities of bile acids would facilitate the liberation of glucose from liver into blood by the activating action of the bile acids.

Administered bile acids may be trapped in liver cells (*18*, *19*), but it has not been ascertained that the acids enter liver cell through cell membrane and act on glucose-6-phosphatase which is located condensedly around the nucleus (*14*). However, other unknown factors may be involved in the mechanism of the hyperglycemia following administration of cholate, such as the damage of the pancreatic islet cells as will be reported in the next paper.

SUMMARY

1. Hyperglycemic response of rabbits to subcutaneous, intravenous and rectal administration of sodium cholate and sodium desoxycholate is described.

2. The elevation of blood sugar level was more intensive in starved animals than in usually fed ones.

3. A decrease of reduced glutathione in blood was observed in the animals with hyperglycemia following the injection of cholate.

The author wishes to express his thanks to Prof. H. Yoshikawa and Dr. M. Nakao for their support and encouragement.

This study has been aided in part by a grant in aid from the Scientific Research Fund of the Ministry of Education.

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l of Biochemistry, Vol. 45, No. 12, 195

STUDIES ON "ALKALIGENIC ACID", A NUCLEIC ACID CONTAINING CONSIDERABLE AMOUNTS OF SUGAR-POLYPEPTIDE COMPLEX ISOLATED FROM A CERTAIN SPECIES OF ALCALIGENES FAECALIS

I. PREPARATION AND CHARACTERIZATION*

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(Received for publication, April 7, 1958)

Recently we have found in a certain species of *Alcaligenes faecalis* a special kind of nucleic acid which is characterized by a high content of sugar-polypeptide complex to which for the sake of convenience has been given a temporary name „ alkaligenic acid". Various species of alcaligenes bacteria were searched for this peculiar substance, resulting, however, in negative results. It was isolated as a copper salt from the free nucleic acid fraction, *i.e.*, the filtrate from the nucleoprotein precipitation of the microorganism. After decomposition with sodium sulfide the copper salt was subjected for further purification to continuous paper electrophoresis which the authors had employed for the preparation of protein free nucleic acids from the nucleoproteins of a variety of bacteria (*1*).

EXPERIMENTALS AND RESULTS

Isolation of Alkaligenic Acid—The bacterial cells were grown in an ordinary broth for 5 days, collected and extracted with ethanol-ether (1 : 1) and then with chloroform. 150 g. of the defatted dry bacteria were ground throughly employing a stainless steel ball mill, and extracted with 2 liters of diluted acetic acid (pH 2.5). The residue from the acetic acid extraction was shaken with 3 liters of phosphate buffer of pH 6.6 for 4 hours. The slightly turbid supernatant fluid separated by centrifugation at 3,000 r.p.m. from the phosphate extract was further centrifuged at 14,000 r.p.m. for 30 minutes in the cold, yielding clear extracts. The combined phosphate extracts were brought to pH 2.4 with glacial acetic acid to give rise to the precipitation of nucleoproteins. To the filtrate from the nucleoproteins was added 20

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* The major part of this work was reported at the 27th Anniversary Meeting of the Japanese Biochemical Society held in Kyoto, April, 1955.

per cent copper chloride until no more precipitation occurred. Upon 30 minute standing, the precipitate of copper salt was separated by centrifugation, washed 3 times with water. The copper salt was suspended in a given amount of phosphate buffer of pH 7.7 which is the same as used for electrophoresis so that the suspension contains about 30 mg. of substance per ml. on a dry weight basis. Then it was decomposed by the addition of 20 per cent sodium sulfide, care being taken that the pH value of the reaction medium does not exceed 8.0. The whole mixture was centrifuged to separate a clear supernatant solution from a dark precipitate of copper sulfide, the latter being washed 3 times with water by centrifugation. The supernatant combined with the washings was concentrated *in vacuo* approximately to the initial volume of phosphate buffer employed. The concentrate was centrifuged at 14,000 r.p.m. for 30 minutes to obtain a clear solution and the latter was subjected to the separation procedure by continuous paper electrophoresis. The chromatogram on paper curtain is indicated in the above figure.

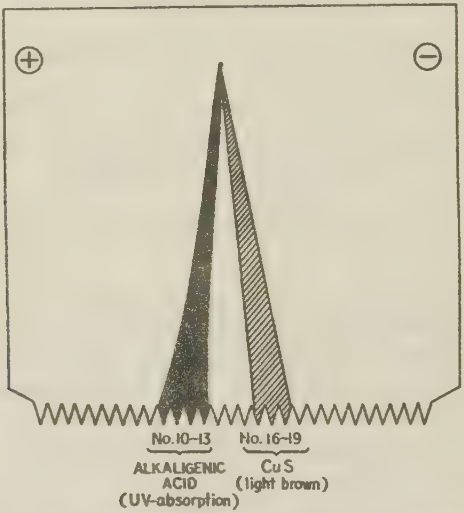


FIG. 1. Separation of alkaligenic acid by continuous paper electrophoresis.

The track run by alkaligenic acid can be traced as a dark zone reaching drip points No. 10–13 when the dried paper is viewed against ultraviolet light through a 253 mμ shortwave filter. The light brown colored zone extending between drip points No. 16–19 is the track presented by CuS which has been retained in solution without forming a precipitate. However, none of ninhydrin positive area has been found anywhere on the paper.

Experimental conditions: phosphate buffer of pH 7.7 and I=0.19; field strength, 400 v; current, 60 ma.; paper, Toyo filter paper No. 5 A, 42×44 cm.; rate of application, 30 mg./1 ml./3 hrs.

The effluents from the drip points corresponding to alkaligenic acid were collected, dialyzed against distilled water in an ice box until entirely free from phosphate ion and the non-dialyzable portion was concentrated in a vacuum desiccator over NaOH and P_2O_5 to a viscous solution. Upon addition of ethanol to the concentrate, the sodium salt of alkaligenic acid precipitated as a colorless fibrous matter. The precipitate was dissolved in water and upon addition of glacial acetic acid to the solution free alkaligenic acid precipitated at pH 1.6–1.8. The product was treated with absolute ethanol and ether to obtain a colorless amorphous substance, which weighed 1.1 g., corresponding to 0.78 per cent on a dry weight basis of defatted bacteria.

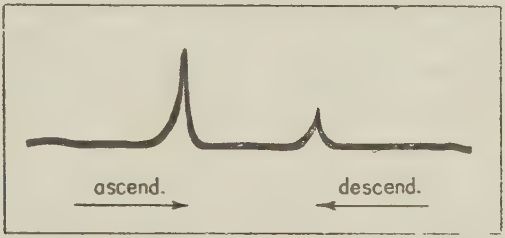


FIG. 2. Electrophoretic pattern of alkaligenic acid. Sample solution, 15 mg. of sample was dissolved in 0.3 ml. of phosphate buffer of pH 6.8 and I=0.1; current, 5 ma./0.3 cm^2.; duration of electrophoresis, 60 minutes.

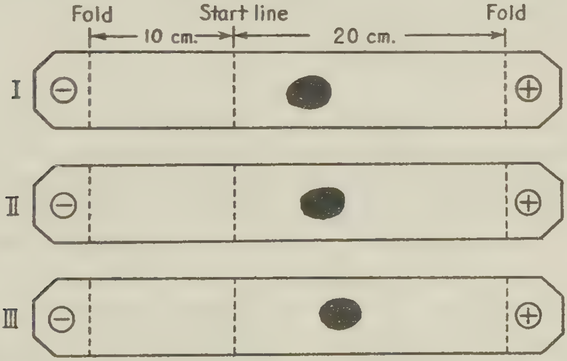


FIG. 3. Paper electrophoresis of alkaligenic acid. The substance moves towards the anode as a single spot visible when viewed against ultraviolet light through a shortwave filter. Samples applied, 100 μg./0.01 ml.; paper, Toyo filter paper No. 51.; applied voltage, 7.5 volts/cm.

I: acetate buffer of pH 5.0 and I=0.14.
II: phosphate buffer of pH 7.3 and I=0.16.
III: borate buffer of pH 8.6 and I=0.08.

Properties of Alkaligenic Acid—The following characteristics were established. N, 13.7 per cent; P, 8.2 per cent. N was measured by an improved

Kjeleldahl-Nessler method (*2*) and P by the Fiske-Subbarow method (*3*). It is difficultly soluble in water. It dissolves in dilute alkali and from the solution it precipitates isoelectrically at pH 1.6–1.8. Fig. 2 and 3 show the electrophoretical homogeneity of this substance.

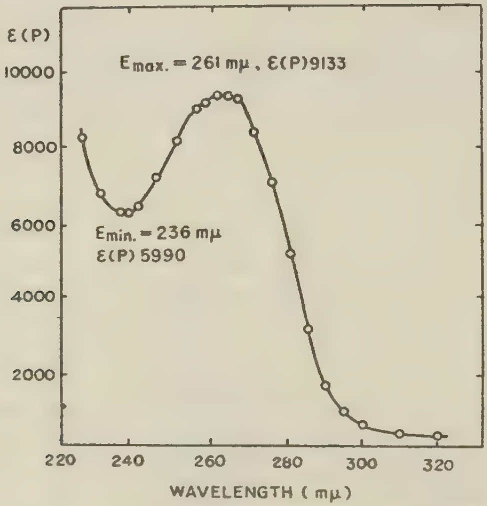


FIG. 4. Ultraviolet absorption spectrum of alkaligenic acid. Test solution, 100 μg. of specimen dissolved in 3 ml. of 0.01 *N* NaOH. Hitachi's spectrophotometer of EPB-U type.

TABLE I

Chemical Tests on Alkaligenic Acid

Reaction	Result	Concentration of test solution (Na salt)	Test for
Ninhydrin*	+	400 *μg./0.01 ml.* (original sample)	Peptide or protein
"	+	100 *μg./0.01 ml.* (total hydrolysate)	"
Biuret	−	1 *mg./0.5 ml.*	"
Protein-chloroform gel**	−	1 *mg./0.5 ml.*	Protein
Orcinol HCl	+	100 *μg./5 ml.*	Ribose
Diphenylamine	+	100 *μg./2 ml.*	Deoxyribose
Urea HCl*	−	1 *mg./0.01 ml.*	Ketose
Ammoniacal $AgNO_3$*	−	500 *μg./0.01 ml.*	Reducing substance
Aniline hydrogen* phthalate	−	500 *μg./0.01 ml.*	"
SO_4 test	−	Ash from 10 *mg.* of sample incinerated	Organic sulfate

* Spot test.

** Sevag's method.

This compound has the ultraviolet absorbing power showing ε (P) 9133 at $E_{max.}$ (261 mμ) and ε (P) 5990 at $E_{min.}$ (236 mμ) as illustrated in Fig. 4. A number of chemical reactions were tested giving the results described in Table I.

Detection of Individual Components

Ribose—1 mg. of material together with 0.1 ml. of 1 *N* HCl was heated in a sealed tube at 100° for 1 hour. 0.01 ml. of the hydrolysate equivalent to 100 μg. of material was subjected to paper chromatography using three different solvent systems, (1) *n*-butanol-acetic acid-water (4 : 1 : 5), (2) phenol-1 per cent ammonia, (3) *n*-butanol-formic acid-water (12 : 1 : 7). A spot was obtained having the identity with D-Ribose in color reactions as well as R_f values.

2-Deoxyribose—10 mg. of material were taken in a small centrifuge tube, to which were added 4.0 ml. of DNase solution containing 2 mg. enzyme per ml. measured by the Kunitz's densitometric method (*4*), 6.0 ml. of acetate buffer of pH 6.0 and 0.4 ml. of 0.05 *M* $MgSO_4$ and the entire mixture was thoroughly mixed and kept at 37° for 10 hours. The reaction mixture was made alkaline to pH 9.0 with ammonia and 2 ml. of Klein's phosphatase solution (*5*) was added and again incubated at 37° for 10 hours. The whole mixture after neutralization was boiled for 10 minutes and centrifuged. The supernatant fluid was deproteinized by the protein-chloroform gel method. The protein-free solution was concentrated to 0.5 ml., brought to pH 1.5 with 2 *N* HCl and heated at 100° for exactly 12 minutes. 0.05 ml. of this solution representing 1 mg. of material was subjected to paper chromatography using three different solvent systems and in each case a spot was revealed by an aniline hydrogen phthalate test as well as a diphenylamine test showing an R_f value identical with that of 2-deoxy-D-ribose from thymus DNA which was similarly treated and simultaneously run.

Detection of Adenine, Guanine, Cytosine, Uracil, and Thymine—3 mg. of material were hydrolyzed in a sealed tube with 0.1 ml. of 70 per cent $HClO_4$ at 100° for 1 hour. The hydrolysate was neutralyzed with conc. NaOH and brought to the total volume of 0.4 ml. 0.02 ml. protions of this solution were applied to paper chromatography which was conducted in the manner described in the previous report (*6*). As shown in Fig. 5, five ultraviolet absorbing spots, having R_f values identical with those of authentic samples of adenine, guanine, cytosine, uracil and thymine were observed. However, none of the spot indicative of 5-methlcytosine was revealed.

For further confirmation each spot on the chromatogram was extracted with 0.1 *N* HCl except for a guanine spot which was extracted with 1 *N* HCl. Respective extract was measured of the wave length of maximum absorption giving the results indicated in the following figure.

Estimation of Molar Ratio of Purines and Pyrimidines—From the quantitative measurement of purines and pyrimidines performed according to the previous work (*6*) the molar ratios of both bases were calculated giving the results shown in Table II.

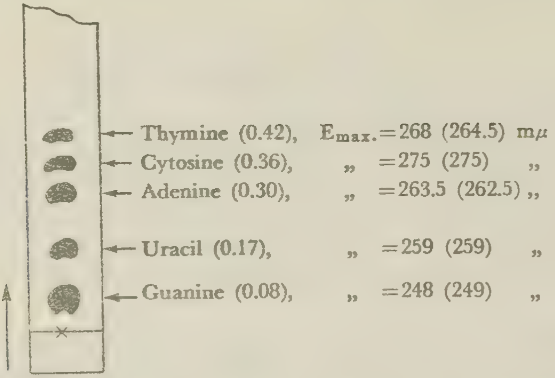

Thymine (0.42), $E_{max.}$=268 (264.5) mμ
Cytosine (0.36), ,, =275 (275) ,,
Adenine (0.30), ,, =263.5 (262.5) ,,
Uracil (0.17), ,, =259 (259) ,,
Guanine (0.08), ,, =248 (249) ,,

FIG. 5. Paper chromatograms of purine and pyrimidine bases isolated from alkaligenic acid. Five UV absorbing spots of nuclein bases are observable, figures in parenthesis denoting R_f values of the individual bases. The values of $E_{max.}$ in parenthesis are those found for authentic specimens. Ascending method. Solvent, *n*-butanol-0.5 *N* NH_4OH (7 : 1). Paper, Toyo filter paper No. 5 A.

TABLE II

Purine and Pyrimidine Contents in Alkaligenic Acid

Nuclein bases	Molar ratio on a P atom basis		Actual % recovery on a N weight basis
	I*	II**	
Adenine	28.8	1.00 } 2.31 Purine	29.4
Guanine	37.2	1.31	38.3
Cytosine	21.3	0.75 } 1.16 Pyrimidine	13.2
Uracil	11.3	0.40	4.7
Thymine	1.7	0.01	0.7
Actual total recovery	76.3***		86.3
Purine : Pyrimidine		2.00 : 1.00	

* Molar ratios of bases per 100 g.-atoms of P in hydrolysate corrected for a 100 per cent recovery.

** Molar ratios of bases expressed relative to the adenine value as 1.00.

*** Sum of the actual values in mole of bases which are not corrected for a 100 per cent recovery.

Quantivative Estimation of DNA and RNA Contents

Since it has been experimentally evidenced that alkaligenic acid contains

2 types of sugar, *i.e.*, D-ribose and 2-deoxy-D-ribose, and 5 nuclein bases such as adenine, guanine, cytosine, uracil, and thymine, it appears likely to suggest that this substance comprises DNA and RNA as its molecular contents. From this viewpoint the attempt has been made to evaluate its DNA and RNA contents. In this evaluation, however, correction was made for observed values of DNA and RNA according to the formula reported in the previous paper (*6*).

DNA—The procedure due to Seibert (*7*) was employed. 2.69 mg. of sample were dissolved in 0.1 ml. of 0.1 *N* NaOH and made up to a total volume of 10 ml. with water. 2.0 ml. of this solution equivalent to 538 μg. of sample or 44.2 μg, of sample P were mixed with 4.0 ml. of diphenylamine reagent and the whole mixture was heated at 100° for 10 minutes. The resulting blue colored solution was measured of its optical density at $E_{max.}=595$ mμ. Since 44.2 μg. of alkaligenic acid P gave 11.8 μg, of DNA-P, the content of DNA-P in the sample is estimated at 26.7 per cent of the total P.

RNA—The determination was conducted by the technique due to Kerr (*8*). 1.0 ml. of the solution the same as used for DNA estimation containing 269 μg. of sample or 22.1 μg. of sample P was diluted with water to 5 ml. and there were added 5 ml. of conc. HCl containing 0.02 per cent $FeCl_3 \cdot 6H_2O$ and 0.3 ml. of 10 per cent orcinol in ethanol. The mixture was heated at 100° for 30 minutes to form a green colored solution, cooled under running tap water and made up with water to a total volume of 15 ml. The optical density of the colored solution was measured at $E_{max.}=670$ mμ. The correction due to the coexistence of DNA which also gives this color reaction to a small extent was made by subtracting 0.158 μg. RNA-P per 1 μg. DNA-P from the total RNA-P value observed. Analysis showed that 22.1 μg. of alkaligenic acid P contained 11.2 μg. of RNA-P, the latter figure corresponding to 50.7 per cent of the total P of the specimen.

Quantitative Estimation of Readily Hydrolyzable Phosphate—3.23 mg. of alkaligenic acid were hydrolyzed with 3 ml. of 1 *N* HCl at 100° for 7 minutes and the hydrolysate was centrifuged. A known volume of the supernatant plus washings was neutralyzed with ammonia using *p*-nitrophenol as indicator and analyzed for P content according to the Fiske Subbarow method. 82 μg. alkaligenic acid P liberated 12.4 μg. P, corresponding to 15.1 per cent of the total P.

Liberation of Polypeptide in Combination with Ribose and Glucosamine

The following three sets of experiments showed the presence of the polypeptide concerned.

Experiment 1 (*7 Minute-1 N HCl Hydrolysis of Alkaligenic Acid*)—15 mg. of material together with 1.0 ml. of 1 *N* HCl was heated in a sealed tube at 100° for 7 minutes and the hydrolysate was centrifuged to remove insoluble matters. The supernatant solution was vacuum evaporated to dryness 3 times with water and the dry residue was dissolved in 0.15 ml. of water.

0.03 ml. aliquots of this solution were subjected to paper electrophoresis, the result of which is given in Fig. 6.

It is worthy of noticing that the polypeptide section 2 has been shown to be free of phosphate by a negative reaction of Hanes-Isherwood, and also free of the reducing radical of sugars by a negative aniline hydrogen phthalate reaction.

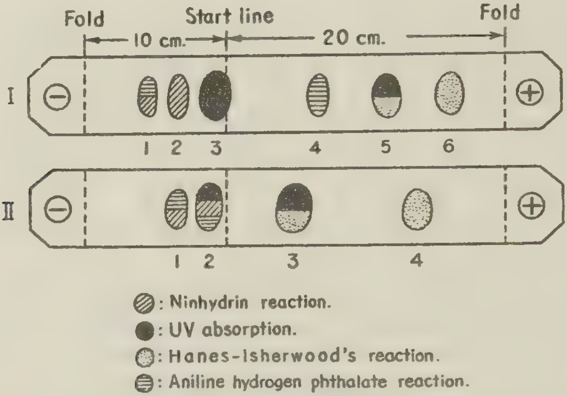


FIG. 6. Paper electrophoresis pattern of 7 minute-1 N HCl-hydrolysate of alkaligenic acid. Both chromatograms I and II show several spots revealed by different dectecting methods.

I: Six spots are visible; (1) glucosamine (ninhydrin reaction, aniline hydrogen phthalate), (2) polypeptide (ninhydrine reaction), (3) adenine, guanine (UV absorption), (4) ribose (aniline hydrogen phthalate), (5) cytidylic acid (UV absorption, Hanes-Isherwood reaction), and (6) H_3PO_4 (Hanes-Isherwood reaction). Experimental conditions; borate buffer of pH 8.6 and I=0.08, 17 volts/cm., 2.3 mA,/cm., 2.5 hours, Tôyô filter paper No. 51.

II: Four spots are observed; (1) glucosamine, (2) polypeptide, adenine, guanine, ribose, (3) cytidylic acid, uridylic acid, and (4) H_3PO_4. Experimental conditions; phosphate buffer of pH 7.3 and I=0.16, 17 volts/cm., 2.1 mA./cm., 2 hours, Tôyô filter paper No. 51.

Experiment 2 (*Partial Hydrolysis of the Polypeptide Section*)—Paper area corresponding to spot 2 in Fig. 6-I, *i.e.*, the polypeptide section was cut off from three sheets of the unsprayed chromatograms obtained in the above. They were combined together, extracted with 15 ml. of N NH_4OH at room tempeature. The extract, after filtering through a glass filter, was desalted by passing through ion exchangers IR-120 and IRA-410, and evaporated *in vacuo* to dryness. The residue was dissolved in 1.0 ml. of 1 N HCl and heated at 100° for 1 hour. The hydrolysate was evaporated *in vacuo* to dryness three times with water in a desiccatar over NaOH and P_2O_5 to remove excess HCl and the residue dissolved in 0.09 ml. of water. 0.01 ml.

aliquots of this solution were applied to paper chromatography in three different solvent systems with the results shown in Fig. 7.

From the R_f values and the color reactions it appears likely that the peptide fraction are separable into three components, ribose, glucosamine, and polypeptide by partial acid hydrolysis. Actual proof for the presence of polypeptide has been established by the following experiment 3.

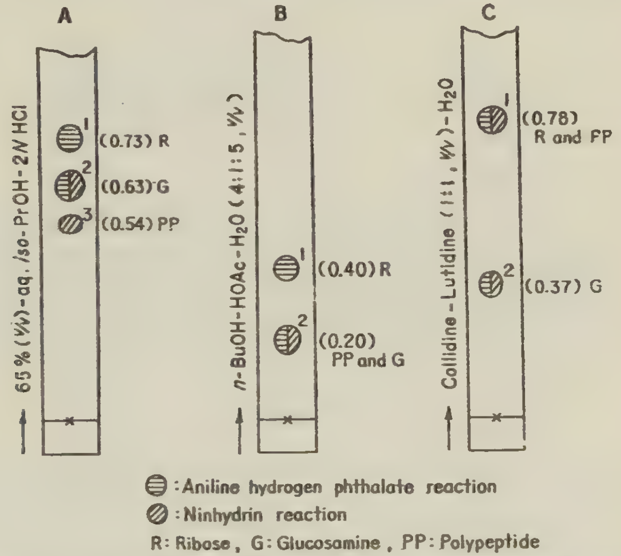

A
B
C
65% (v/v)-aq. iso-PrOH-2N HCl
n-BuOH-HOAc-H2O (4:1:5, v/v)
Collidine-Lutidine (1:1, v/v)-H2O
1 (0.73) R
2 (0.63) G
3 (0.54) PP
1 (0.40) R
2 (0.20) PP and G
1 (0.78) R and PP
2 (0.37) G
⊜ : Aniline hydrogen phthalate reaction
▨ : Ninhydrin reaction
R: Ribose, G: Glucosamine, PP: Polypeptide

FIG. 7. Paper chromatograms of 1 hour-1 *N* HCl-hydrolysate of the polypeptide fraction extracted from the paper area corresponding to spot 2 in Fig. 6-I. They were obtained by the ascending method using three different solvent systems. Figures in parenthesis denote individual R_f values. It is remarkable that all the spot 1 in the three chromatograms gave a reddish coloration with aniline hydrogen phthalate characteristic of pentose, while each spot 2 gave a brown coloration with the same reagent.

Experiment 3 (*Total hydrolysis of the Polypeptide Section*)—A) Paper section containing spot 3 in Fig. 7-A or spot 1 in Fig. 7-C was cut off from eight sheets of the unsprayed chromatograms. The combined paper cutouts were extracted with 15 ml. of 0.1 *N* NH_4OH, the extract, after desalting through ion exchangers, was evaporated *in vacuo* to dryness and the residue was hydrolyzed in a sealed tube with 0.5 ml. of 6 *N* HCl at 100° for 20 hours. From the hydrolysate, excess of HCl was removed by repeated evaporation with water in a desiccator over NaOH and P_2O_5, and the final residue was dissolved in 0.08 ml. of water. A 0.05 ml. portion of the solution was applied to paper electrophoresis, giving rise to various amino acids of neutral, acidic and basic nature, as shown in Fig. 8.

B) Paper section including spot 2 in Fig. 7-B was cut off from eight sheets of the unsprayed chromatograms, and the extract from the cutouts was treated in a similar manner to the above and subjected to paper electrophoresis. In this case in addition to amino acids glucosamine was detected, the data of which are illustrated in Fig. 9.

The three sets of experiments 1, 2, and 3 mentioned above demonstrate that 7 minute hydrolysis of alkaligenic acid with 1 *N* HCl at 100° liberates a ninhydrin positive substance and this substnace by further hydrolysis with 1 *N* HCl at 100° for 1 hour separates into ribose, glucosamine and polypeptide.

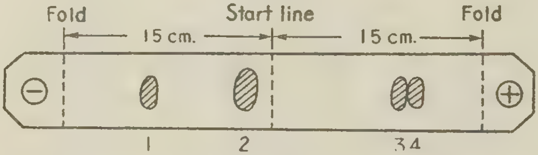


FIG. 8. Paper electrophoresis pattern of the total hydrolysate of the extracts from the paper area of ninhydrin positive spot 3 in Fig. 7-A or spot 1 in Fig. 7-C. Four ninhydrin positive spots are illustrated; (1) lysine, (2) neutral amino acids, (3) glutamic acid, and (4) aspartic acid.

Experimental conditions: phosphate buffer of pH 7.3 and I=0.16; field strength, 17 volts/cm.; time of electrophoresis, 1.5 hours; paper, Tôyô filter paper No. 5 A (3×40 cm.).

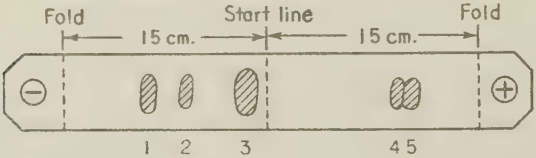


FIG. 9. Paper electrophoresis pattern of the total hydrolysate of the extracts from the ninhydrin positive spot 2 in Fig. 7-B indicating five ninhydrine positive spots; (1) lysine, (2) glucosamine, (3) neutral amino acids, (4) glutamic acid, and (5) aspartic acid.

Experimental conditions: the same as described in Fig. 8.

Quantitative Estimation of the Total Amino Acids Present in Alkaligenic Acid—The procedure was the same as in the previous report (*1*). 9.71 mg. of sample gave 82.8 μg. of amino acids, leucine serving as a standard. Thus the quantity of ninhydrin positive compounds including amino acids and glucosamine was found to be 8.53 per cent.

Detection of Individual Amino Acids in the Total Hydrolysate of Alkaligenic Acid by Paper Chromatography—10 mg. of sample were hydrolyzed in a sealed tube with 1.0 ml. of 6 *N* HCl at 100° for 20 hours. The hydrolysate, after removing excess HCl by repeated evaporation *in vacuo* over NaOH and P_2O_5 was taken up in 0.05 ml. of 0.005 *N* HCl and the whole solution was subjected to two-dimensional paper chromatography. The result is indicated in Fig. 10.

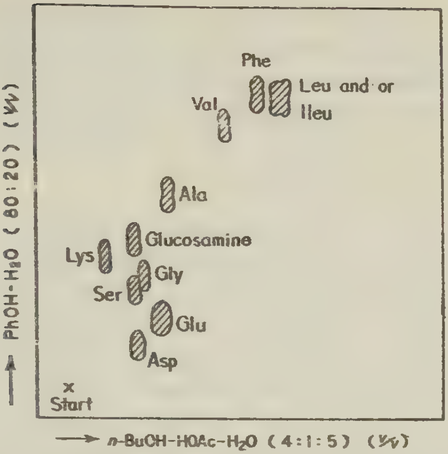


FIG. 10. Two-dimensional paper chromatogram of the total hydrolysate of alkaligenic acid showing 9 or 10 amino acids and glucosamine. Spray reagent, 1 per cent ninhydrin in *n*-butanol-phenol (90:10); paper, Tôyo filter paper No. 51.

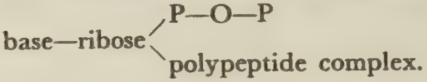
DISCUSSION

It has been evidenced that alkaligenic acid is high molecular compound showing considerable homogeneity by the Tiselius electrophoresis method and consisting of three principal components of DNA, RNA and polypeptide. It contains polypeptide to such a high degree as 8.53 per cent on a dry weight basis. The polypeptide fraction which is of rather labile nature can be liberated in an appreciable quantity by 7 minute hydrolysis with 1 *N* HCl at 100°. The liberation of polypeptide increases to a certain extent through continued hydrolysis up to about 1 hour, after which it is accompanied by the splitting of amino acids from the polypeptide.

Report has been made in the previous paper (*1*) that nucleic acid associated with polypeptide have been isolated from several microorganisms employing the same method and the content of polypeptide estimated was as low as approximately 1 per cent.

The most characteristic features which differentiate alkaligenic acid from those microbial nucleic acids are; (1) its markedly higher content of polypeptide, (2) liberation of polypeptide in combination with ribose and glucosamine by mild acid hydrolysis with simultaneous liberation of inorganic phosphate. The sequence order and the manner in which these three components, polypeptide, ribose and glucosamine, are linked together and further with nucleic acid moiety are problems remaining to be elucidated by future studies. In the present state of our research no detailed information is available as to the linkage order of these components. However, theoretically three types of linkage order are possible; (1) ribose-polypeptide-glucossamine, (2) ribose-glucosamine-polypeptide, (3) polypeptide-ribose-glucosamine. Concerning the linking of this polypeptide complex with the nucleic acid moiety the presence of readily hydrolyzable phosphate bond should be taken into account. Assuming the presence of pyrophosphate bond it is very likely to suggest two types of linkage:

(a) base—ribose—P—O—P—polypeptide complex

(b) 

$$\text{base—ribose}\begin{cases}\text{P—O—P}\\ \text{polypeptide complex.}\end{cases}$$

The former is a type conforming to the type of uridine diphosphate-glucosamine polypeptide involving labile phosphate (*9*). The latter belongs to a type in which the polypeptide and the nucleotide part may possibly possess a ribose molecule in common. Anyway, there seems to be no doubt that the polypeptide complex is attached to the RNA instead of the DNA moiety, on the ground that it possesses ribose as constituent and also that in the deoxyribose part of the DNA moiety there remains no free OH radical available for conjugation with the polypeptide complex. Combination of pyrophosphate group with the hydroxyl radical of serine present in the polypeptide which is considerably stable to acid hydrolysis may be excluded, since the polypeptide fraction liberated by 7 minute—1 *N* HCl hydrolysis was free of phosphate as stated before.

With regard to the nucleic acid part of alkaligenic acid a few remarks will be given. As has already been indicated in Table II, the actual recovery in mole of nuclein bases on a P atom basis was found to be 76.3 per cent. The value seems to be significantly low compared with that obtained with ordinary nucleic acids, which the latter shows usually more than 96 per cent. In terms of percentage recovery on a N weight basis the actual recovery of the bases gave a value of 86.3 and this value will exceed 95 if the polypeptide content of alkaligenic acid, *i.e*,. 8.5 per cent is considered in recovery calculation. These data may imply that the molar concentration of bases is somewhat lower with respect to the atomic concentration of P in the alkaligenic acid molecule, thus hereby too providing a favorable support for a pyrophosphate bond.

The composition of bases seems to resemble that of the nucleic acids obtained from the nucleoprotein fraction of *Alcaligenes* of the same strain in that both are of a G-C type and charactirized by an exceedingly high content of guanine and an exceptionally low content of thymine.

As to the biological significance of this unusual type of nucleic acid nothing can be said, but a mere speculation in the present state of study. This substance has been isolated from a special strain of alcaligenes bacteria. This may be an abnormal product formed in the microorganism due to the deranged metabolism of nucleic acid induced either by an intrinsic or by an extrinsic factor. Here is one conjecture that this organism might be a variant strain involving a prophage and alkaligenic acid be a compound having genetically some relationsphip with the latter. The fact that both alkaligenic acid and the nucleic acid occurring in the same cells were pronouncedly poor in thymine, points to the possibility that an increased demand for thymine might have been imposed on by some prophage production, thus bringing about the diminution of thymine content of the nucleic acid produced within the cells involved.

SUMMARY

1. In a certain species of *Alcaligenes faecalis* has been found to occur a special kind of nucleic acid referred to as alkaligenic acid. It is a high molecular compound of N 13.7 per cent and P 8.2 per cent, showing considerable homogeneity in electrophoresis.

2. It contains about 8.5 per cent of sugar-polypeptide complex in a bound form which can be liberated by 7 minute—1 *N* HCl—hydrolysis with simultaneous splitting of inorganic phosphate. The complex includes ribose, glucosamine and polypeptide, the latter comprising about 10 amino acids.

3. The nucleic acid moiety is composed of both types of nucleic acids, 26.7 per cent of DNA-P and 50.7 per cent of RNA-P of the total P and are characterized by a significantly high content of guanine and a markedly low content of thymine.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

THE MECANISMS OF CATECHINS METABOLISM

II. NEUTRAL SUBSTANCES IN THE URINE OF RABBITS ADMINISTERED (+)-CATECHIN

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(Received for publication, April 18, 1958)

In the previous paper (*1*), authors reported that protocatechuic, vanillic and 3-hydroxybenzoic acids were found as intermediary metabolites o catechin in the acidic fraction of the urine of rabbits administered (+)-catechin.

In this experiment, three substances were detected by paper partition chromatography in the neutral fraction of the urine. The isolation of these substances was thus performed using a large amount of urine of rabbits, administering a large amount of catechin, and two substances were isolated in crystalline forms, having m.p. 105-106° (G) and 145-146° (H) respectively. Another substance (F) was, however, unable to obtain as a crystalline state, because of its small quantities.

From the results of elemental analysis and estimation of molecular weight, H was shown its molecular formula as $C_{11}H_{12}O_4$. Color reaction and alkali hydrolysis indicated the presence of 3,4-dihydroxyphenolic group and a lactone ring. Thus δ-(3,4-dihydroxyphenyl)-γ-valerolactone (II) was postulated for H. On the otther hand, G was shown as $C_{11}H_{12}O_3$ and its chemical properties were investigated as the same methods as in H, and chemical structure was posturated to be δ-(3-hydroxyphenyl)-γ-valerolactone (I) for G.

These substances were considered as intermediary metaolites of catechin and the precursors for protocatechuic, 3-hydroxybenzoic and vanillic acids which were reported in the previous paper.

EXPERIMENTAL AND RESULT

Paper Partition Chromatography—After removing acidic substances by shaking the ether extracts with sodium bicarbonate solution as reported in the previous paper, the ether layer was dried with anhydrous sodium sulfate.

Ether was then removed and the residues were tested on paper partition chromatography using a filter paper of Toyo Roshi No. 50 and the mixture solution of water saturated benzene and acetic acid (9:1 by volume).

The colors were developed by spraying with a solution of diazotized sulfanilic acid and then followed 20 per cent aqueous sodium carbonate solution by Bray *et al.* (*2*). Control experiments were carried out in the same method. Three spots wera observed by chromatography for the urine administered catechin, whereas no spot was observed in control. The R_f values and color reaction of these neutral substances were shown in Table I.

TABLE I

Spots	Color reactions	R_f values
F	Pink	0.96
G	Yellow	0.75
H	Red-purple then black-brown	0.14

Isolation of Neutral Substances in Crystalline States—To isolate the substances corresponding to spots of F, G and H in the paper chromatography described above, 20 rabbits (2–3 kg. weight) were given 0.6 g of catechin per 1 kg. of body weight a day for one week with Okara, and 32.4 litets of urine was collected by previous method. The urine was saturated with sodium chloride, extracted once with an equal volume of ethyl acetate and then with a half volume. Each ethyl acetate layer was combined, dried with anhydrous sodium sulfate and concentrated to 500 ml. The solution was then shaken with 30 per cent aqueous ammonium carbonate solution, by which acidic substances were separated, dried with anhydrous sodium sulfate and evaporated. An amorphous residue was obtained. On keeping over night at room temperature, it partially solidified. To this fraction 50 ml. of ether was added and the residues were mixed well with ether. By filtration the crystalline substances were separated and washed with a small amout of ether. The crystalls were decolorized with charcoal and recrystallized from water. Recrystallization was repeated from a mixture of ethanol and benzene (1 : 9 by volume). White needle crystalls were obtained, m.p. 145-146°. Yield 0.6 g. The supernatant was evaporated and residues were decolorized with charcoal. Recrystallization was repeated from water and then from benzene in the same way. White plate crystals were obtained, m.p. 105-106°. Yield 1.2 g.

The substance of spot F was however not isolated in crystalline state because of its small quantities, even using a column chromatography with cellulose powder.

Rotation—These compounds were optically active like as (+)-catechin. $[\alpha]_D^{16°}$; G=32.8°, H=25.7°. (in absolute ethanol)

Molecular Weights—Results (by K. Rast's method) were shown in Table II.

Elemental Analysis—Results were shown in Table III.

TABLE II

Crystals	Sample (*mg.*)	Campher (*mg.*)	T (°*C*)	Molecular weight
G	0.217	5.205	8.7	187
	0.193	3.955	9.8	194
H	0.170	3.450	9.5	202
	0.225	3.815	11.0	207

k=39.0

TABLE III

Crystals	Sample (*mg.*)	CO_2 (*mg.*)	H_2O (*mg.*)	C (%)	H (%)
G	3.960	9.993	2.210	68.87	6.25
	3.262	8.208	1.834	68.67	6.29
H	4.151	9.687	2.191	63.69	5.91
	3.477	8.147	1.843	63.94	5.93

Molecular Formula—The molecular formulas were given for G and H as $C_{11}H_{12}O_3$ and $C_{11}H_{12}O_4$ respectively, from the results of molecular weight determination and elemental analysis shown in Table IV.

TABLE IV

Formular	M. W.	C (%)	H (%)
$C_{11}H_{12}O_3$	192.12	68.76	6.25
$C_{11}H_{12}O_4$	208.12	63.45	5.81

Color Reactions—The neutral substances gave color reactions with diazotized sulfanilic acid and sodium carbonate as described, above, and this fact indicated that they belonged to phenolic compounds. The substances were tested moreover specific color reactions for ortho-diphenolic compounds as follows;

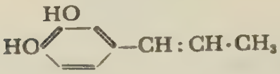
Crystalline substance H.

a) Ferric chloride; Blue-green color was demonstrated and then turned brown. By the addition of alkaline solution, it turned red.

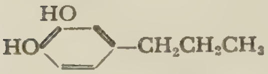
b) Phosphomolybdic ammonium; Red color was shown with aqueous solution.

c) Phosphomolybdic acid; The color was blue. By the addition of aqueous ammonium solution, it turned blue-green.

d) Alkaline solution; The color was red, indicating



or



type. (*3*).

e) Precipitates with lead acetate; By addition of concentrated lead acetate solution, precipitates appeared (*4*).

f) Fichten Span reaction; The reaction was negative, indicating no phloroglucin ring.

Crystalline substanc G.

It showed no such specific reaction of 3,4-dihydroxyphenolic compound as crystalline H. It seemed to be a monohydroxyphenolic compound. No phloroglucin ring was detected.

Presence of Lactone Ring—These substances were dissolved in excess $N/10$ sodiumhydroxyde solution by heating and after cooling sodium hydroxyd remained was titrated with $N/10$ sulphuric acid. One mole of alkali was consumed in both cases. By heating with conc. alcoholic ammonia solution these gave acid amides. These facts indicated the presence of lactone ring.

The lactone hardly gave anilide and phenylhydrazide by treatment with aniline and phenylhydrazine respectively, thus the formula of the lactone may be considered γ-lactone like nature.

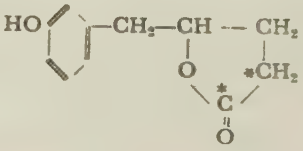
DISCUSSION

From the results of elemental analysis and the estimation of molecular weight, the crystalline G was considered as $C_{11}H_{12}O_3$ and H as $C_{11}H_{12}O_4$. In addition, color reactions suggested that G is a monohydroxyphenolic and H is a 3,4-dihydroxyphenolic compounds. Concerning the substitution position of hydroxyl group in G, 3-position was likely considered, scince a large amout of 3-hydroxybenzoic acid was excreted by the administration of catechin and 3-hydroxyphenolic compounds known to be derived from 3,4-dihydroxy phenolic compounds (*5*).

Two other oxygens were considered to form, a lactone ring, and the lactone seemed to be γ-lactone, considering from its stability and the chemical structure of these two compounds, were thus similar and the difference was probably only due to the number of hydroxyl group.

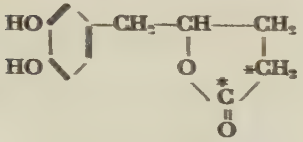
From the results obtained, following chemical structure were thus posturated;

G=



(I)

H=



(II)

considering from their chemical structure, the benzene rings of these substances which found in the urine seemed to be derived from the side ring of catechin. As reported in the previous paper, the side benzene ring of catechin is relatively stable, whereas pyron and phloroglucine rings tend easily to rupture. The carbons marked with in above formula are considred to be derived from the carbon of phloroglcin ring.

The chemical structures of F, G and H have been further investigated in detail in our laboratory.

SUMMARY

1. From the urine of rabbits administered (+)-catechin, three neutral substances (F, G and H) were recognized by paper partition chromatography and these substances regarded as intermediary metabolites of catechin.

2. Amoung these three substances, G and H were isolated in crystalline states and their chemical structure were posturated as δ-(3-hydroxyphenyl)-γ-valerolactone and δ-(3,4-dihydroxyphenyl)-γ-valerolactone respectively.

The authors are grateful to M. Shido for his kind helps of elemental analysis.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

GLUCOSE METABOLISM AND AMINO ACID IN BRAIN SLICES

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(Received for publication, April 18, 1958)

It is a generally believed fact that glucose is the main energy supplying substrate for the brain activity. Glucose is thought to be metabolized through the glycolytic route and the tricarboxylic acid cycle in brain slices which are prepared without disrupting the majority of cell structures and represent rather truly the metabolism in living brain. Recently, Beloff-Chain *et al.* (*1*) found that the uniformly labelled C^{14}-glucose metabolized in brain slices is partly converted into amino acids, of which glutamic acid is quantitatively the most important.

In the present work the distribution of C^{14} from uniformly labelled glucose in brain slices under the various conditions including the influence of some inhibitors, was studied using the radio-paperchromatography technique. Glutamic acid was thought to function possible as a gate to the amino acids to be formed from glucose, because glutamic acid was most resistant to inhibitors.

MATERIALS AND METHODS

Animals and Incubation—Guinea pigs, weighing 200–300 g. were used throughout. Glucose uniformly labelled with C^{14} was obtained from the Radiochemical Centre, Amersham, England. The specific activity was about 60 μc/mg., varying slightly from batch to batch. All chemicals used were reagent grade and were used without further purification. Preparation of cerebral cortex slices and conditions of incubation were almost the same as those previously described (*2, 3*). About 100 mg. of brain slices were incubated under pure oxygen in 1.5 ml. of the phosphate medium containing 100 mg. per cent of glucose. To each vessel 10 μc of C^{14}-glucose was added. The incubation was 90 minute duration at 37°. At the end of the incubation period, the brain slices were removed from the incubating vessels, blotted of filter paper and then rapidly transferred to 1 ml. of 75 per cent ethanol and homogenized in a Potter's homogenizer. Aqueous ethanolic extract was prepared according to Awapara (*4*).

Paperchromatography and Radioautographs—0.13–0.15 ml. samples of the extract, corresponding accurately to 50 mg. of brain slices, were transferred to sheets (40×40 cm.) of Toyo filter paper No. 51 (Toyo Roshi Kaisha, Ltd.) by means of a special micropipette, drying immediately by a current of warm air from a hair-drier. 0.05 ml. samples of the incubation medium were similarly transferred to sheets of 4×40 cm. The solvent employed for onedimensional development of samples of the incubation medium was solvent 1. Two-dimensional chromatograms were run first in solvent 1 and then in solvent 2, or first in solvent 3 and then in solvent 4. The chromatograms were run in all-glass vessels at 20°. The following solvents were used (*1*).

Solvent 1: *n*-butanol 40 ml., acetic acid 10 ml., water 50 ml.
Solvent 2: *tert.*-butanol 80 ml., picric acid 4 g., water 20 ml.
Solvent 3: *n*-butanol 40 ml., ethanol 20 ml., water 10 ml.
Solvent 4: phenol 80 ml., water 20 ml.

After the developed chromatograms were allowed to dry, they were placed in contact with a X-ray film (Fuji industrial X-ray safety film, type 200, high contrast, non-screen type) in a specially made light-tight frame for 3–5 days according to the solvents used. The films were developed by a high contrast developer (Fuji Rendol) for 5 minutes at 20°.

RESULTS

Fig. 2 and 3 show the typical radioautographs obtained from an extract of brain slices after aerobic incubation with C^{14}-glucose in the phosphate medium. Each spot of the chromatogram was identified by comparing its R_f value with that obtained with pure samples of known substance run in the same conditions and also by referring to its reaction with ninhydrin. As shown by Beloff-Chain *et al.* (*1*), the spots corresponding to glutamic acid, glutamine, γ-aminobutyric acid, aspartic acid and alanine were detected. It seemed that glutamic acid was quantitatively the most predominant.

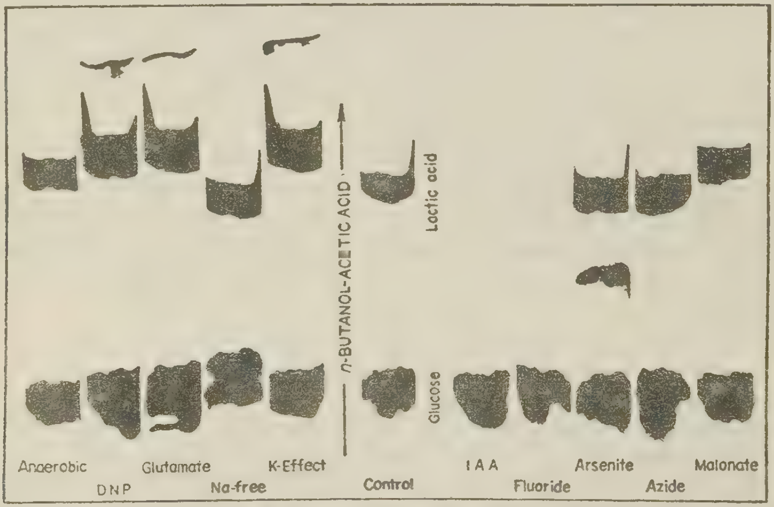

FIG. 1. Radioautograph of sample of the medium after incubating brain slices with C^{14}-glucose under various conditions.

L-Glutamic acid, KCl or inhibitor was added at a following concentration (per liter): 2,4-Dinitrophenol (DNP), $5\times10^{-5}\,M$; Glutamate, $10^{-2}\,M$; K-Effect, $10^{-1}\,M$ KCl; Iodoacetic acid (IAA), $10^{-4}\,M$; Fluoride, $10^{-2}\,M$; Arsenite, $5\times10^{-5}\,M$; Azide, $10^{-3}\,M$; Malonate, $10^{-2}\,M$.

The glucose metabolism of brain slices is very much affected by varying the composition of the incubation medium. Addition of 10^{-1} *M* KCl causes a pronounced increase in the oxygen uptake, the glucose consumption and the lactic acid formation (*3*). The increase of lactic acid formation is easily seen in Fig. 1. The influence of the addition of 10^{-1} *M* KCl on the distribution of C^{14} is demonstrated in Fig. 4. The formation of the free amino acids from glucose by brain slices also seemed to be increased; especially glutamine was much more formed in K-rich medium. Sodium-free condition showed a similar effect on amino acid formation.

As shown in previous work (*5*, *6*), the addition of L-glutamic acid (10^{-2} *M*) or 2,4-dinitrophenol (DNP) (5×10^{-5} *M*) also increases the matabolism of brain slices. Glutamic acid (10^{-2} *M*) did not suppress, but rather enhanced slightly the formation of amino acids. DNP, at the concentration used (5×10^{-5} *M*), decreased clearly the formation of amino acids, in spite of increasing the glucose consumption and the lactic acid formation (Fig. 7). But the formation of glutamic acid and γ-aminobutyric acid was rather resistant to DNP.

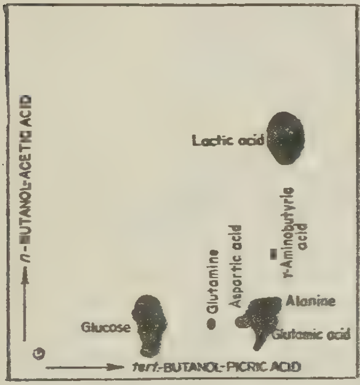

FIG. 2. Radioautograph of aqueous ethanolic extract of brain slices incubated aerobically with C^{14}-glucose.

The chromatogram was developed first in *n*-butanol-acetic acid and then in *tert.*-butanol-picric acid.

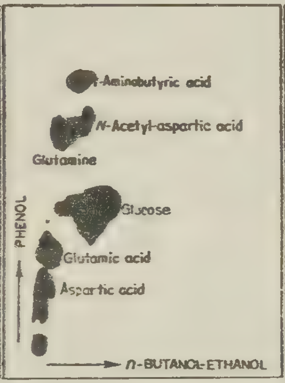

FIG. 3. The same as Fig. 2.

The chromatogram was developed first in *n*-butanol-ethanol and then in phenol.

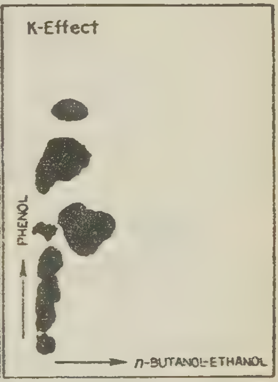

FIG. 4. The effect of adding KCl to the medium at a concentration of 10^{-1} *M*.

The chromatographic technique was the same as Fig. 3.

Under anaerobic condition it was found that the lactic acid formation was very much increased (Fig. 1), and on the two-dimensional chromatogram, there was no spot besides glucose and lactic acid.

Effect of some metabolic inhibitors on brain slices was previously studied by measuring not only the oxygen uptake, but also the glucose consumption and the lactic acid formation (*6*). The metabolism of brain slices was sensitive to many known inhibitors of the glycolytic route and the citric acid cycle, at a rather low concentration. The effect of some of them on the formation of amino acids was shown in Fig. 5 and 6. Iodoacetic acid (IAA) (10^{-4} *M*) and fluoride (10^{-2} *M*) reduced the lactic acid formation; the spot of lactic acid was not detected almost completely (Fig. 1). Arsenite (5×10^{-5} *M*), azide (10^{-3} *M*), and malonate (10^{-2} *M*) increased the formation of lactic acid (Fig. 1). Under the influence of arsenite, the spot of pyruvic acid was found. In generally speaking, the formation of amino acids besides glutamic acid was very sensitive to metabolic inhibitors. In the presence of IAA, fluoride or azide at a concentration presently used, no spot was found besides that of glutamic acid and of aspartic acid. Malonate and arsenite did not inhibit completely the formation of γ-aminobutyric acid.

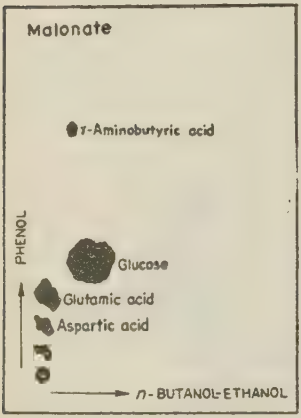

FIG. 5. Radioautograph of aqueous ethanolic extract of brain slices showing the effect of malonic acid (10^{-2} *M*).

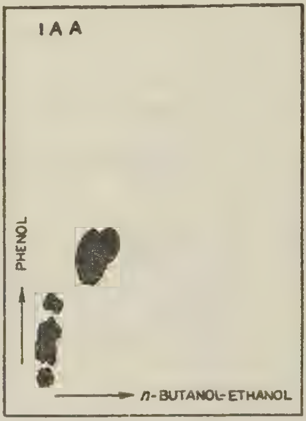

FIG. 6. Radioautograph of aqueous ethanolic extract of brain slices showing the effect of iodoacetic acid (10^{-4} *M*).

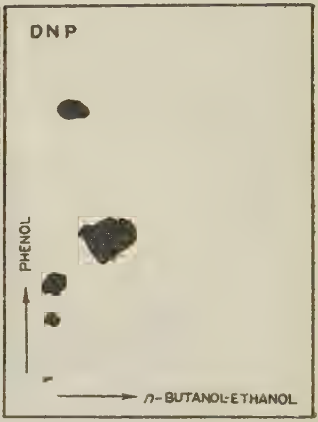

FIG. 7. Radioautograph of aqueous ethanolic extract of brain slices showing the effect of 2,4-dinitrophenol (5×10^{-5} *M*).

One hour after injecting C^{14}-glucose intracysternally, the guinea pig was killed and the extract of brain tissue was prepared as usual. The radioautographic pattern was almost the same as that obtainen *in vitro* after incubation. From these animals the brain slices were prepared as previously described and incubated without further addition of C^{14}-glucose in the presence of inert glucose at a 10^{-2} *M* concentration or in the absence of substrate. In conformity with previous findings (*2*), the spot of glutamic acid was much reduced after incubation without substrate comparing with that after metabolizing inert glucose as substrate.

DISCUSSION

According to the investigations by Beloff-Chain *et al.* (*1*), the amino acids are not formed at all in muscle from glucose after incubation under similar conditions, and there is a striking difference between the *in* *vitro* pattern of glucose metabolism in muscle and that in brain. The formation of amino acids from glucose would be a peculiar phenomenon to brain tissue. They also pointed out the importance of glutamic acid; first, glutamic acid was quantitatively the largest, and second, it was already formed after only 3 minute incubation. In the present work many of the findings of Beloff-Chain *et al.* (*1*) were confirmed.

Amino acids would be formed in brain slices through the aerobic metabolism of glucose by means of an energy-coupling mechanism. Amino acids were not formed under anaerobic condition, and were much reduced in the presence of DNP (Fig. 7). Addition of KCl at a concentration of 100 mM increased the P^{32} incorporation into phosphate fractions of brain slices (*5*). It also accelerated the formation of amino acids as shown in Fig. 4. These observation suggested the necessity of energy-coupling mechanism for the amino acid formation in brain slices. Many known inhibitors of glucose metabolism also inhibited the formation of amino acids, although some of them increased the glucose utilization and the lactic acid formation. Among amino acids formed from glucose, glutamic acid seemed to be most resistant to the action of inhibitors. This observation, together with the findings by Beloff-Chain *et al.* (*1*) on the importance of glutamic acid, suggested a central position of glutamic acid in brain metabolism.

Glutamic acid might function as a gate for the formation of amino acids from glucose in brain slices, that is, it might play a connecting role between the metabolism of glucose and of amino acid. These considerations are of interest in relation to a general belief that the concentration of glutamic acid in brain is changed reflecting the functional state of brain (*7*). Also, glutamic acid is thought to be a possible source of ammonia which is more formed in the excited conditions of animals, basing upon the analyses of ammonia formation in brain slices. Previous observation (*2*) on the relation between glutamic acid content and ammonia formation in brain slices, obtained from the chemical analysis, was confirmed from the present data, obtained using the slices labelled *in vivo*.

The fact that the distribution of C^{14} in amino acids in brain of animals injected intracysternally with C^{14}-glucose was similar to that obtained *in vitro* with brain slices, shows the usefulness of brain slices as an experimental preparation for analysing the metabolism in living brain.

SUMMARY

1. The distribution of C^{14} from uniformly labelled C^{14}-glucose incubated aerobically with brain slices for 90 minutes was studied using the radio-paperchromatography technique.

2. Effects of various conditions and some metabolic inhibitors which change the glucose metabolism of brain slices upon the pattern of radio-autograph was investigated.

3. The formation of amino acids from plucose was clearly inhibited by metabolic inhibitors of the glycolytic route and the citric acid cycle.

4. Glutamic acid was thought to function possibly as a connecting gate between glucose metabolism and amino acids in brain tissue.

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c Journal of Biochemistry. Vol. 45. No. 12. 1958

INFLUENCES OF γ-AMINOBUTYRIC ACID AND SUBSTANCES POSSESSING SIMILAR CHEMICAL STRUCTURE ON HEXOKINASE OF THE BRAIN AND HEART MUSCLE

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(Received for publication, June 6, 1958)

Hexokinase is considered to be one of the most important enzymes involved in the sugar and the energy metagolism in various organs and tissues owing to the fact the monosaccharides join in the metabolic current only when this sugar is converted to phosphate ester after receiving terminal phosphate from ATP by the catalytic action of hexokinase; and since glucose is the sole source of energy in the brain (*1–11*), the importance of the enzyme hexokinase is worthy of a special consideration (*11*).

Hexokinase of the brain, as has already been pointed out by us (*11*), differs from hexokinase of other tissues and organs not only in specificity against substrate but also in the position (*13*) of phosphorylation; and reports about several other properties differing from those of hexokinase in other organs have been made by various investigators. Among these reports, Long (*14*) describes that the hexokinase activity of the brain is far greater than hexokinase acitvity of any other organ. This point is, as stated previously, extremely interesting when it is considered in connection with the fact that the brain relies solely on glucose as its main source of energy.

On the other hand, γ-aminobutyric acid, being one of amino acids, is now believed to exist in free state in the brain and to have an intimate relationship with function of the brain, as described reviews by Roberts (*15*) and by us (*16*). As for the action of γ-aminobutyric acid besides its power to accelerate tissue respiration, its influences on enzymes such as acetylcholine metabolism (*19*), histaminase (*20*), and tyramine oxydase (*21*) are known. In this connection, I have studied the acceleration of hexokinase activity in the brain and heart muscle by γ-aminobutyric acid as well as by the substrances possessing a similar chemical structure, and having obtained somewhat satisfactory results in clarifying chemically and structurally these actions on enzyme, the results are described herewith.

MATERIALS AND METHODS

Experimental Materials—The cerebral cortex and the heart muscle of normal rabbits weighing 2–3 kg. (male and female) were employed as experimental materials.

Experimental Substances—γ-aminobutyric acid manufactured by Daiichi Kagaku Yakuhin K. K. was recrystallized several times and used for the experiment (m.p. 201°). By my own method (*22*) γ-amino-α-hydroxybutyric acid was synthesized from γ-aminobutyric acid with the melting point of 191–192°. γ-amino-β-hydroxybutyric acid was donated by Prof. Okumura of the Department of Psychiatry, Okayama University Medical School. *N*-acetyl-γ-aminobutyric acid was synthesized by my method (*22*) (m.p. 126–7°). δ-amino-*n*-valeric acid was synthesized by Wallach's (*23*) method (m.p. 154°). β-Alanine of m.p. 197° (decomp.) of Ishizu Seiyaku, Ltd., and *n*-butylamine of Wako Pure Chemical Industries, Ltd. (b.p. 77°) respectively were used.

Determination of the Hexokinase Activity—The determination of the hexokinase activity was carried out by Long's method (*14*). Namely, 1.0 per cent KCl homogenate containing 10 per cent tissue was added to experimental substances, 0.2 ml. of 0.01 *M* glucose, 0.4 ml. of 0.02 *M* ATP, 0.1 ml. of 1 *M* NaF, 0.1 ml. of 0.2 *M* $MgCl_2$, 0.5 ml. of 0.2 *M* Tris-HCl buffer (pH 7.0) and 0.4 ml. of additional substance or 0.4 ml. of water with 1.0 ml. of brain or heart muscle homogenate, and these were incubated at 37.5° exactly for 20 minutes. Then after stopping the reaction with $Ba(OH)_2-ZnSO_4$ (*24*) and eliminating protein at the same time, the quantity of glucose contained in filtrate was measured by Somogi-Nelson's method (*25, 26*) and the amount of glucose consumed in the reaction was calculated.

RESULTS

Influences of γ-Aminobutyric Acid on the Brain and the Heart Muscle Hexokinase—Using γ-aminobutyric acid at various concentrations of 0.001, 0.005, 0.025, and 0.100 *M*, its action on hexokinase of the brain and the heart muscle was examined (the data show the average value of 8 cases). It has been found that γ-aminobutyric acid at the concentration of 0.001 *M* shows approximately a ten pen cent increase in the hexokinase activity but no further increase in the rate of the activity can be observed at the other higher concentrations in the brain. On the other hand, when γ-aminobutyric acid is added to the heart muscle, whose normal value of hexokinase activity

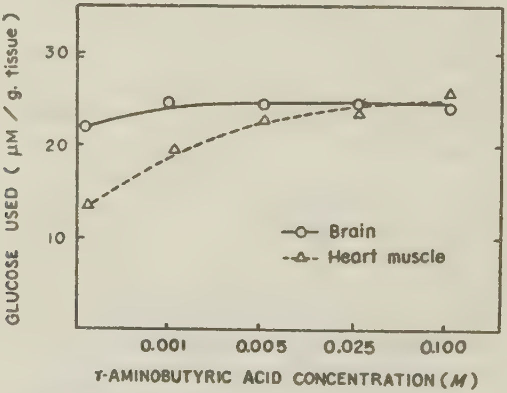

FIG. 1. Influences of γ-aminobutyric acid on the brain and the heart muscle hexokinase.

is about 60 per cent of that of the brain, is accelerated quite markedly; and the hexokinase activity increases to 1.5 times that of the brain at the concentration of 0.001 *M* and as the concentration is increased more, and the activity reaches almost twice that of the brain. As for the glucose consumption in one gram of each tissue, no difference can be observed at the concentration beyond 0.025 *M* in the values between the brain and the heart muscle.

Influence of γ-Aminobutyric Acid and the Substances Having Analogous Chemical Structures on the Brain and the Heart Muscle Hexokinase—With the use of substances possessing a similar chemical structure such as *N*-acetyl-γ-aminobutyric acid, γ-amino-α-hydroxy-butyric acid, γ-amino-β-hydroxy-butyric acid, δ-amino-*n*-valeric acid, and *n*-butylamine each at the concentration of 0.025 *M*, influences on hexokinase activity has been studied.

TABLE I

Experiments with the Brain

Names of compound used	No. of expt's	Rate of increase (av.)
Control	9	1.00
γ-Aminobutyric acid	9	1.10
N-Acetyl-γaminobutyric acid	8	0.74
γ-Amino-α-hydroxybutyric acid	6	0.95
γ-Amino-β-hydroxybutyric acid	3	1.04
δ-Amino-*n*-valeric acid	8	0.99
β-Alanine	7	0.94
n-Butylamine	8	1.29

TABLE II

Experiments with the Heart Muscle

Names of compounds	No. of expt's	Rate of increase (av.)
Control	9	1.00
γ-Aminobutyric acid	9	1.69
N-Acetyl-γ-aminobutyric acid	7	0.44
γ-Amino-α-hydroxybutyric acid	6	0.98
γ-Amino-β-hydroxybutyric acid	3	0.94
δ-Amino-*n*-valeric acid	4	2.05
β-Alanine	6	0.99
n-Butylamine	5	1.22

Influences of N-Acetyl-γ-aminobutyric Acid on the Brain and the Heart Muscle Hexokinase—*N*-Acetyl-γ-aminobutyric acid has been sythesized by me primarily for the purpose of clarifying chemical-structurally the accelerating action of γ-aminobutyric acid on hexokinase activity, and using this newly synthesized compound, changes in the hexokinase activity according to variations in the concentration of the compounds have been pursued. As the results, the average of six cases is shown in Fig. 2, and the hexokinase activity is inhibited in both the brain and heart muscle though the rate of inhibition is lesser in the brain.

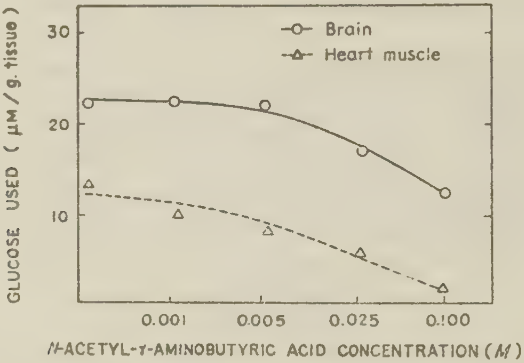

FIG. 2. Influences of *N*-acetyl-γ-aminobutyric acid on the brain and the heart muscle hexokinase.

DISCUSSION

The importance of ATP and Mg^{++} in the enzymatic activity of hexokinase is apparent, as hexokinase becomes active only in the presence of these two and the same can be said of hexokinase in general. Long (*14*) reported on the influences of pH value, the ATP and fluoride concentrations on hexokinase activity; and we (*27*) investigated the influences of the fluoride, calcium ions, glutamic acid, and aspartic acid concentrattion on the hexokinase activity of the brain and reported about the accelerating actions of glutamic acid and aspartic acid on the brain hexokinase activity.

As shown in the experimental results, γ-aminobutyric acid accelerates the hexokinase activities both in the brain and the heart muscle but the rate of acceleration is slight in the brain while it is marked in the heart muscle. In this series of experiments, bacause γ-aminobutyric acid seems to be contained in a considerable quantity in the brains of animal and its content in other tissues appears to be extremely slight or none at all (*16*), the reason why γ-aminobutyric acid does not accelerate so apparently the activity of the brain hexokinase seems to lie in the fact that as γ-aminobutyric acid exists in a considerable quanity in the brain itself and has already accelerated the activity under normal conditions, the normal brain maintains a high level of the activity. Thus in the experiment of addition of this acid, a slight amount added seems to be sufficient to raise immediately the acceleration to the maximal value. It may be for this reason that the maximal value of acceleration of hexokinase activity in the brain practically coincides with the maximal in the heart muscle.

Next, as key in clarifying the action of γ-aminobutyric acid on hexokinase, the similar action of the substances possessing chemical structure akin to γ-aminobutyric acid has been investigated. When -OH group is led into the place of the α- or β-position of γ-aminobutyric acid as shown in Table I and II, the power accelerating hexokinase activity is lost. Moreover, when *N*-acetyl-γ-aminobutyric acid in which one of hydrogens in the amino-group is replaced by acetyl-group is added, hexokinase activity is markedly inhibited in the cases of the brain and beart muscle. The effects of these concentrations are shown in Fig. 2. In this case, the rate of inhibitory action is less in the brain, and in the heart muscle even when the concentration is such that the hexokinase activity is expected to be inhibited markedly, the normal level of the activity is maintained continuously. The enzyme system of the brain receives less influences from the chemical agent (γ-aminobutyric acid) than other tissues, and the same can be said of influences of other agents as can be seen in Tables I and II; and it is quite interesting to notice that the brain activity is of such a nature as to resist well against shock. γ-Aminobutyric acid is C amino acid, but on examining actions of β-alanine and δ-amino-*n*-valeric acid, which are C_3 and C_5-amino acids, it has been revealed that δ-amino-*n*-valeric acid hardly affects the brain hexokinase in any degree whereas in the case of the heart muscle it acts as to accelerate its activity quite markedly. β-Alanine, on the other hand, does not affect at all either the brain or the heart muscle. Since most substances being handled here are amino acids, it may seem inconsistent to bring up *n*-butylamine which is an amine possessing chemical characteristics different from amino acid, nevertheless, it may be mentioned here that *n*-butylamin derived from γ-aminobutyric acid by replacing its carboxyl group with methyl group, acts on the hexokinase activity quite strikingly.

As it is readily understandable from the foregoing experimental results the accelerating action on hexokinase is not solely possessed by γ-aminobutyric acid, but δ-amino-*n*-valeric acid which is C_5-amino acid likewise possesses a similar accelerative power; and taking *n*-butylamine into consideration similarly this accelerating action of certain chemicals on hexokinase may in all likely be attributable to the group, NH_2-CH_2-CH_2-CH_2-.

SUMMARY

In the present experiment concerning the action of both γ-aminobutyric acid and substances possessing similar chemical structure upon the hexokinase activity of the brain and the heart muscle, the following conclusions were drawn.

1. γ-aminobutyric acid acceleates the brain-hexokinase activity slightly while it increases the hexokinase activity of the heart muscle quite markedly.
2. γ-Amino-α-hydroxybutyric acid as well as γ-amino-β-hydroxybutyric acid do not accelerate the hexokinase activity.

3. *N*-Acetyl-γ-aminobutyric acid inhibits the hexokinase activity of the brain and the heart muscle quite strikingly.

4. β-Alanine does not accelerate the hexokinase activity in the brain and the heart muscle, but δ-amino-*n*-valeric acid promotes the hexokinase activity in the heart muscle.

5. *n*-Butylamine possesses the power to increase the hexokinase activity.

6. It is assumed that the accelerating power of γ-aminobutyric acid on the hexokinase activity is due to the NH_2-CH_2-CH_2-CH_2-group.

I gratefully acknowledge the valuable guidance of Prof. D. Jinnai in preparation of this manuscript.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

ACTIVATION AND INHIBITION BY BIVALENT METAL IONS OF YEAST GLYCYLGLYCINE DIPEPTIDASE*

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(Received for publication, June 30, 1958)

The fact that various peptidases require metal ions as activators for their reactions is now generally construed as being due to the necessity of metal ions as mediators of the complex formation between the enzyme and substrate. This concept has been developed by Smith and his co-workers, on the basis of their experiments with various dipeptidases isolated from animal tissues (*1*, *2*, *3*). Smith has further suggested that the primary reaction in the activation process consists in the combination of the metal ion with the enzyme protein. An alternative theory in this respect has been presented by Najjar for some metal activated enzymes. He assumed that the metal ion first forms a complex with the substrate, and the resulting metal-substrate complex serves as true substrate for the enzyme (*4*). The theory was, however, criticized by Smith as implausible, at least in the case of leucine amino peptidase (*3*), which was one of the main subjects of his own studies.

The present investigation was undertaken with a view to obtaining information as to the mode of action of metal ions in the activation of dipeptidase which had been isolated from yeast cells. It was revealed that the enzyme requires specifically Co^{++} ion as the activator and that other bivalent ions such as Cd^{++}, Cu^{++}, Mn^{++}, and Zn^{++} are not only incapable of substituting Co^{++} ion, but are more or less inhibitory towards the action of the enzyme. By kinetic analysis it was deduced that Co^{++} ion functions as an effective bridging agent between the enzyme and substrate and that the inhibitory effects of other bivalent ions are due to their competitive action with Co^{++} ion.

MATERIALS AND METHODS

Preparation of the Enzyme—The dipeptidase was isolated from yeast cells by the following procedure. Two pounds of baker's yeast were macerated with ethylacetate and mixed with an equal weight of water. The mixture was adjusted to pH 6.0 by the addition of

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* This paper was read at the 28th Annual Meeting of the Japanease Biochemical Society in 1955.

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KOH and after being covered with toluene, allowed to autolyze at room temperature with occasional shaking. After 5 hours the mixture was centrifuged and the precipitate was resuspended in water and left to stand for further 30 hours to continue autolysis. The supernatant obtained by centrifugation was cooled and brought to 0.4 saturation with solid ammonium sulfate. The precipitate formed was discarded and the clear solution was brought to 0.6 saturation by further addition of ammonium sulfate. The precipitate formed was collected by suction, dissolved in 80 ml. of water, and after adjusting the pH of the solution to 6.0, acetone was added to 33 volume per cent. The mixture was then subjected to centrifugation, and to the supernatant obtained, acetone was added up to 48 volume per cent. The precipitate formed anew was dissolved in 40 ml. of acetate buffer (pH 6.0, 0.1 *M*), and heated for 3 minutes at 60° in the presence of $CoCl_2$ (0.01 *M*). A large mass of precipitate produced was removed by centrifugation and to the supernatant was added an equal volume of acetone. The resulting precipitate which contained the enzyme was dissolved in 40 ml. of water and the solution was dialyzed against running water. Most of the experiments in this study were performed with the enzyme preparations at this stage of purification*. The activity of this solution was proved to be stable for several weeks when stored in a refrigerator.

Measurements of Enzyme Activity—The substrate used was glycylglycine ; the concentration applied was 0.05 *M* unless otherwise specified. The reaction mixture was buffered with vernol (0.05 *M*). The enzyme activity was measured by the formol titration method. The activity of the enzyme preparation in the presence of 0.001 *M* $CoCl_2$ was expressed in terms of proteolytic coefficient (C_0) computed according to the formula:

$$C_0 = K_0/E$$

where K_0 is the reaction rate calculated on the basis of per cent hydrolysis per minute, and E is the protein concentration in mg. protein-N per ml. of reaction mixture. The C_0-value of the enzyme preparation used in the experiment was of the order of 250**.

RESULTS AND DISCUSSION

Activity and Stability of Glycylglycine Dipeptidase as Influenced by Various Experimental Conditions—The stability of the dipeptidase was found to be largely influenced by the presence of Co^{++} ions in the medium. Fig. 1 shows the results of an experiment in which the enzyme solution was heated at 60° for 2 minutes in the presence or absence of $CoCl_2$. The pH in the heating mixture was adjusted with acetate or borate buffer (0.05 *M*) to various values indicated in the figure. After cooling, the pH of the mixture was readjusted to 7.8 with veronal buffer and the enzyme activity was assayed. The enzymatic activity was computed as percentage of original activity. These results show that Co^{++} at certain concentration obviously enhances the stability of yeast glycylglycine dipeptidase.

The optimum pH for the hydrolysis of glycylglycine lies at pH 7.7–8.0 (see Fig. 2), which is identical with that reported by Smith *et al.* for glycylglycine dipeptidase from animal source (*1*).

Effect of Various Bivalent Metals—The results of the tests with various metal ions at 0.001 *M* revealed that a marked activation of the enzymatic activity was brought about by Co^{++} (see Table I). It is to be noted that Mn^{++} which has been reported by Smith *et al.* as an activator of glycylglcine dipeptidase from human uterus (*1*), had no effect at all in this case. In the presence of Hg^{++}, the activity was somewhat improved, but this effect seemed to be due to the presence of another enzyme (requiring Hg^{++} as

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* Further purification, although with poor yields, could be performed by fractionating this preparation with acetone of 33 to 48 volume per cent in concentration.

** The C_0-value of the crude autolysate obtained at earlier stage of enzyme preparation was only about 10.

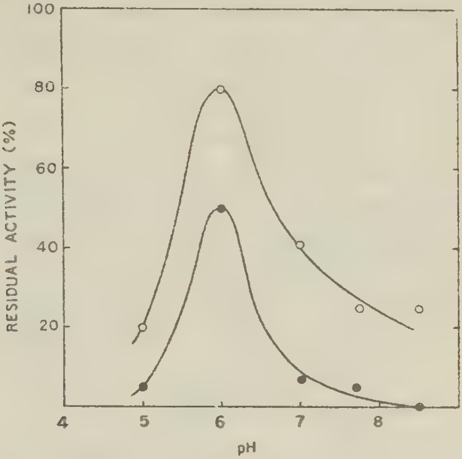

FIG. 1. Stability of glycylglycine dipeptidase as a function of pH. Enzyme solution was heated at 60° for 2 min. in the presence (○) or absence (●) of CoCl$_2$ (0.001 *M*) at various pH Residual activity was expressed as percentage of original activity. Enzyme assay was performed at 35° and pH 7.8 with 0.05 *M* glycylglycine as substrate in the presence of Co^{++} (0.001 *M*).

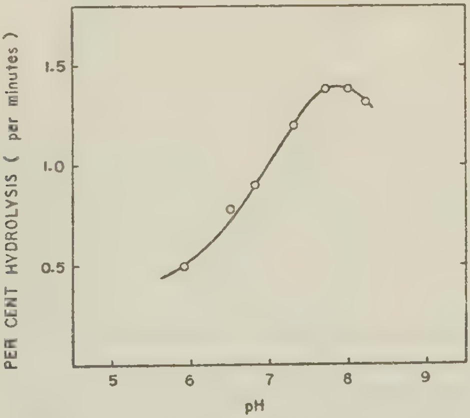

FIG. 2. Effect of pH on enzymatic hydrolysis of glycylglycine. Each reaction mixture contained 0.05 *M* glycylglycine and 0.001 *M* CoCl$_2$. The enzyme concentration was 7 μg. protein-N per ml. reaction mixture.

an activator) in this preparation. In fact, the enzyme preparations that had completely lost (by prolonged storage) the original capacity for glycylglycine hydrolysis (as tested in the presence of Co^{++} as the activator) still showed an activity of hydrolyzing the same substrate, if Hg^{++} was added to the reaction mixture. We shall reserve a detailed investigation of this contaminating dipeptidase for some future occasion.

TABLE I

Effect of Bivalent Metal Ions on the Activity of Glycylglycine Dipeptidase

Tests were performed at 35° and pH 7.8 in the presence of metal ion (0.001 M) and substrate (0.05 M). The enzyme concentration was 8 μg. protein-N per ml. of reactiod mixture.

Metal ion	Per cent hydrolysis per minute (%)
Co^{++}	1.900
Ba^{++}	0.006
Ca^{++}	0.004
Cd^{++}	0.000
Cu^{++}	0.004
Fe^{++}	0.040
Hg^{++}	0.700
Mg^{++}	0.004
Mn^{++}	0.002
Ni^{++}	0.002
Zn^{++}	0.002
None	0.002

Reaction Rate as Influenced by Co^{++} and Substrate Concentrations—The influence of Co^{++} concentration on the enzyme activity was investigated in the presence of fixed concentrations of substrate (0.0125 M–0.2 M). The reaction was initiated by adding substrate to the buffered enzyme solution containing $CoCl_2$ as activator. No prior incubation of enzyme and metal was found to be necessary, since the maximal activation was attained instantaneously. The results are presented in Fig. 3, in which reaction rates are plotted against logarithms of Co^{++} concentrations. The same data are replotted in Fig. 4, showing reaction rate as a function of substrate concentration. As will be seen from the figures, the reaction rate increased with increasing concentration of substrate and activator. With higher concentrations of either component, there was more or less marked retardation of the reaction as will be considered in a later section.

Mechanism of Enzyme Reaction—The experimental facts presented above may accounted for by assuming the following reactions occurring in sequence or in parallel. In these formulae E is the enzyme, S the substrate, A the activator (Co^{++}) and EAS is a complex of the enzyme and substrate formed through bridging by the activator A. The formation of this triple complex, which is assumed to be the essential step for the accomplishment of the enzyme reaction, is thought to be possible in two ways: either by reaction of the EA complex with the free substrate S, or by reaction of the free

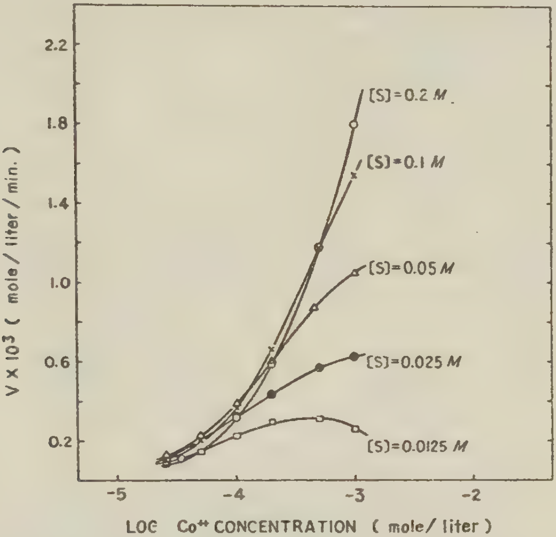

FIG. 3. Activity of glycylglycine dipeptidase as a function of Co^{++} concentration at various substrate levels ([S]). Experiments were performed at 35° and pH 7.8. The enzyme concentration was 9 μg. protein-N per ml. reaction mixture.

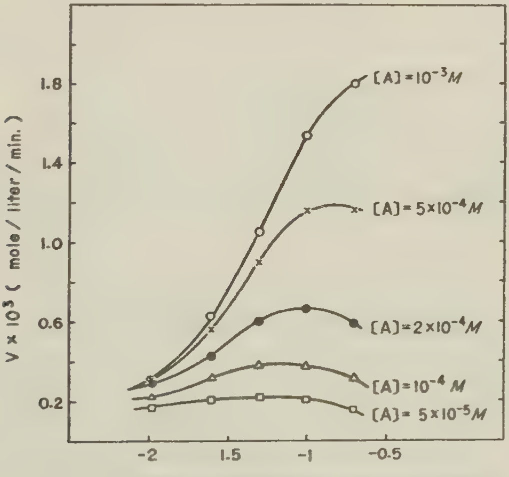

FIG. 4. Activity of glycylglycine dipeptidase as a function of substrate concentration at various levels of activator ([A]).

In the calculation of the quantity β' in the above expression, the above-derived values for K_2 and k [e] were used. The value for K_3 was assumed to be 10^{-3} moles/liter based on the experimental results of Monk (*5*), who

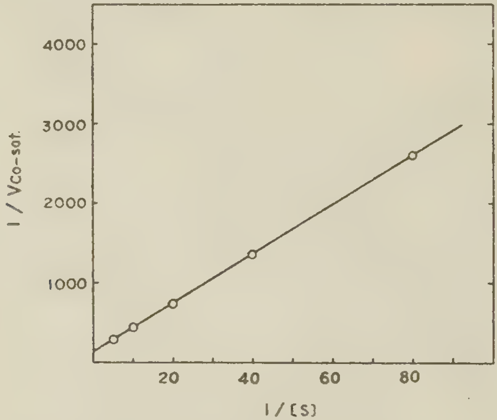

FIG. 6. Reciprocal of Co^{++}-saturated velocity ($1/V_{\text{Co-sat}}$) as a function of reciprocal of substrate concentration.

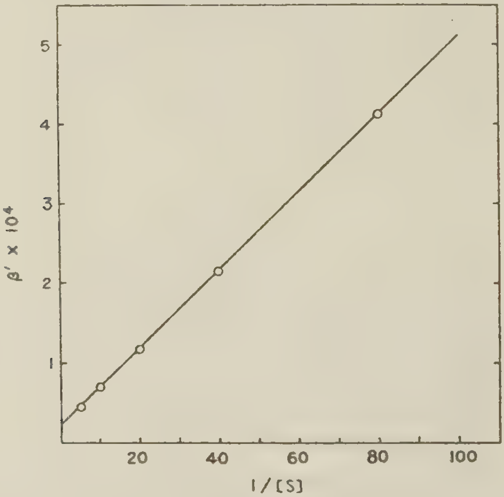

FIG. 7. Linear relationship between β' and reciprocal of substrate concentration. β' was calculatd according to Eq. (11) as described in the text.

investigated the combination of various amino acids and peptide with various metal ions. By plotting β' against 1/[s] (Fig. 7), a straight line was obtained in harmony with the formula (Eq. 11). Based on this result, the values for K_1 and K_5 were computed to be as follows:

$$K_1=4.8\times10^{-6}\text{ moles/liter}$$
$$K_5=2.2\times10^{-1}\text{ moles/liter}$$

The above-stated facts will be more clearly illustrated by the curves shown in Figs. 8, 9, and 10. In Figs. 8 the experimental data for the reaction rate *V*, as plotted against the logarithms of activator concentrations, show a satisfactory agreement with the theoretical curves drawn according to Equation (7), except for the case of activator concentrations higher than 10^{-3} *M*.

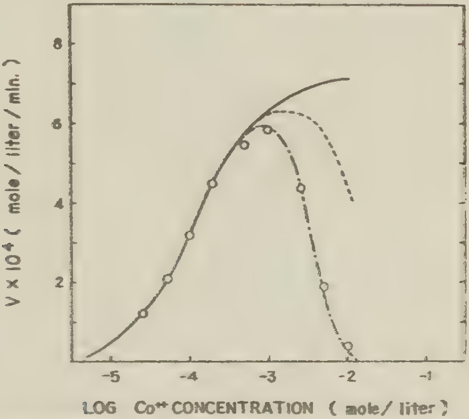

FIG. 8. Inhibitory effect of higher concentrations of Co^{++}. The experiments were performed at 35° and pH 7.8 in the presence of 0.025 *M* of glycylglycine as substrate. The enzyme concentration was 9 μg. protein-N per ml. reaction mixture. Points represent the experimental data. Solid line, dashed line, and broken line were drawn according to Eq. (7), (3), and (14), respectively.

The coincidence with the theory will be more convincingly demonstrated by the data presented in Fig. 9, in which the reaction rates are expressed in terms of ratio to Co^{++}-saturated velocities ($V_{\text{Co-sat.}}$) which were computed from the data in Fig. 5 (see Equation 7′). The points corresponding to higher concentrations of Co^{++} are omitted in Fig. 9. The curves in the figure are drawn according to the theory, *i.e.* Equation (12), as transformed into a formula of first order sigmoid curve:

$$\frac{V}{V_{\text{Co-sat.}}}=\frac{[\mathrm{a}]}{[\mathrm{a}]+\phi}$$

$$\phi=\frac{K_1 K_2}{K_3 K_5}\cdot\frac{([\mathrm{s}]+K_5)\,([\mathrm{s}]+K_3)}{[\mathrm{s}]+K_2} \tag{12}$$

It is apparent form the above foumula, that the half Co^{++}-saturated velocity ($V/V_{\text{Co-sat.}}=1/2$) is reached when the concentration of activator is equal to ϕ, which represents the *apparent dissociation constant* of Co^{++} ion in combination with the enzyme system.

In Fig. 10 are compared the experimental and theoretical values (calculated by Eq. 8) pertaining to $V_{\text{Co-sat.}}$ as a function of substrate concentration. Here again, the agreement may be regarded as satisfactory. The highest level of this $V_{\text{Co-sat.}}$-curve represents the maximum velocity,

$V_{max.}$, and the middle point of the sigmoid curve corresponds to the dissociation constant K_2 of the active complex, EAS (Eq. 8).

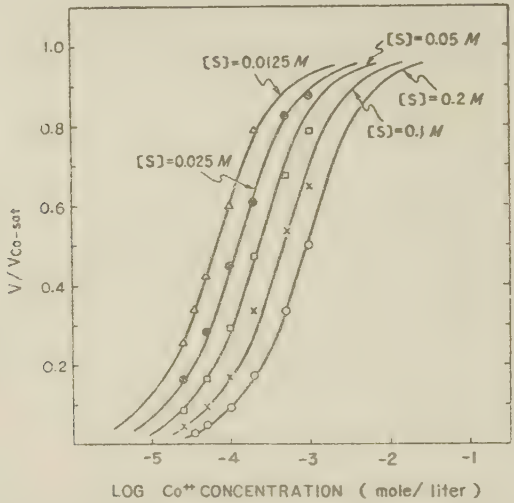

FIG. 9. Ratio of reaction velocity(V) to Co^{++}-saturated velocity ($V_{Co\text{-}sat.}$), as a function of Co^{++} concentration.

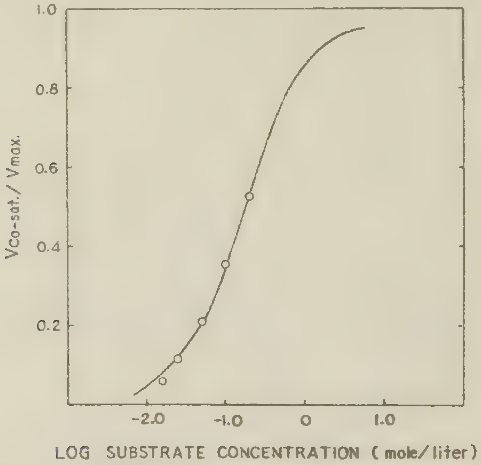

FIG. 10. Ratio of Co^{++}-saturated velocity ($V_{Co\text{-}sat.}$) to maximum velocity ($V_{max.}$), as a function of substrate concentration.

With still higher concentrations of activator, a marked depression of enzymatic activity was noticed (see Fig. 8). The actual extent of depression was found to be much more pronounced than that expected from the above theory (Eq. 3). The deviation of the experimental results in this respect from the theory will be accounted for by assuming another reaction of higher order between enzyme and metal ion, entailing the formation of an inactive complex, EAA_2:

$$EA+2A \underset{}{\overset{K_6}{\rightleftarrows}} EAA_2 \tag{13}$$

where K_6 is the dissociation constant of the complex EAA_2. The equation (Eq. 3) for the reaction rate V will then be modified as follows:

$$V=\frac{k\,[e]}{1+\frac{K_2}{[S]}+\frac{K_1\,K_2}{[S]\,[A]}+\frac{K_1\,K_2}{K_5\,[A]}+\frac{K_2\,[A]^2}{K_6\,[S]}} \tag{14}$$

Using the numerical values obtained earlier for the constants K_1 K_2, and K_5, the value for K_6 in Equation (14) was estimated to be 2×10^{-8} moles/liter. The theoretical values of V obtained by applying these values of K's to Equation (14) are shown by the broken line in Fig. 8, which also agree well with the experimental results.

Inhibition by Bivalent Metal Ions Other than Co^{++}—The effects of various metal ions other than Co^{++} upon the enzyme activity to be displayed in the presence of 0.05 M glycylglycine and 0.001 M Co^{++} were investigated. Among the various ions tested, Cd^{++}, Cu^{++}, Zn^{++}, Mn^{++}, and Mg^{++} were found to inhibit the reaction, whereas Ni^{++}, Fe^{++}, Ca^{++}, and Ba^{++} did not show any noticeable effect. With the former class of inhibiting ions, the functional relationship between the degree of inhibition (H) and the concentration of inhibitor was found to be in the form (see Fig. 11):

$$\begin{aligned} H&=1-\frac{V_{me}}{V_0}\\ &=\frac{1}{1+\frac{\phi_{me}}{[Me]}} \end{aligned} \tag{15}$$

where V_{me} and V_0 represent the reaction rates in the presence and absence, respectively, of the inhibitor ions; [Me] is the concentration of the metal ion, and ϕ_{me} is a constant corresponding to the concentration of inhibitor causing 50 per cent inhibitions.

In another set of experiments, the dependence of the degree of inhibition at fixed concentrations of each inhibitor (Cd^{++} 10^{-5} M, Mn^{++} 10^{-3} M, Zn^{++} 10^{-5} M, and Cu^{++} 10^{-4} M) upon the concentration of the activator Co^{++} was investigated. From Fig. 12, in which the reciprocals of the velocities observed are plotted against reciprocals of Co^{++} concentration, it will be seen that all the straight lines meet at one point on the vertical axis of the graph. As may be shown by the following kinetic consideration, this fact indicates that all the inhibitory metal ions investigated acted competitively with the activator Co^{++} ion.

It may be assumed that the inhibiting metal ion, Me^{++}, can form complexes both with the enzyme and with the substrate as follows:

$$\begin{aligned} &E+Me \overset{K_{me}}{\rightleftarrows} EMe, \qquad && K_{me}=\frac{[E]\,[Me]}{[EMe]}\\ &Me+S \overset{K_{me}'}{\rightleftarrows} MeS && K_{me}'=\frac{[Me]\,[S]}{[MeS]} \end{aligned} \tag{16}$$

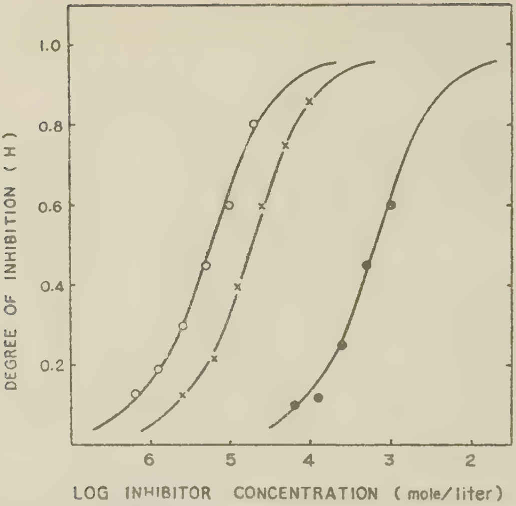

FIG. 11. Inhibition by bivalent metal ions (1). Each test solution contained 0.05 *M* glycylglycine, 0.001 *M* $CoCl_2$, and varied amounts of inhibitor. Experiments were performed at 35° and pH 7.8. (○) Cd^{++}, (×) Zn^{++}, (●) Mn^{++}.

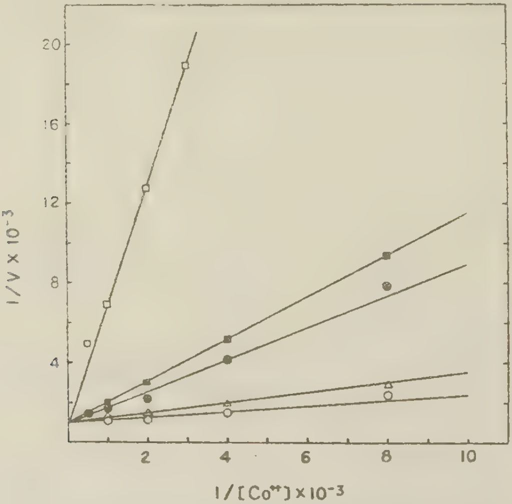

FIG. 12. Inhibition by bivalent metal ions (2). Reciprocal of reaction velocity as a function of reciprocal of Co^{++} concentration. The experiments were performed at 35° and pH 7.8 in the presence of 0.05 *M* glycylglycine. (○) without inhibitor, (□) 10^{-5} *M* Cd^{++}, (■) 10^{-3} *M* Mn^{++}, (●) 10^{-5} *M* Zn^{++}, (△) 10^{-4} *M* Cu^{++}.

where K_{me} and K_{me}' represent the dissociation constants of the respective complexes. Taking these reactions into account, the equation for the reaction rate (V_{me}) in the presence of inhibitor ion, Me^{++}, may be written as follows (*cf.* Eq. 7):

$$V_{\mathrm{me}}=\frac{k\,[\mathrm{e}]}{1+\frac{K_2}{[\mathrm{s}]}+\frac{K_1K_2}{[\mathrm{s}]\,[\mathrm{a}]}+\frac{K_1K_2}{K_3\,[\mathrm{a}]}+\frac{K_1K_2}{K_5\,[\mathrm{a}]}+\frac{K_1K_2\,[\mathrm{s}]}{K_3K_5\,[\mathrm{a}]}+\frac{[\mathrm{Me}]}{K_{\mathrm{me}}}\cdot\frac{K_{\mathrm{me}}'}{K_{\mathrm{me}}'+[\mathrm{s}]}\left(\frac{K_1K_2}{K_3\,[\mathrm{a}]}+\frac{K_1K_2}{[\mathrm{a}]\,[\mathrm{s}]}\right)} \tag{17}$$

Using this equation and Equation (7), we can formulate an equation analogous to Equation (15), in which the inhibition constant ϕ_{me} assumes the form:

$$\phi_{\mathrm{me}}=\frac{K_i\left(1+\frac{K_1K_2}{[\mathrm{a}]\,K_3}+\frac{K_1K_2}{[\mathrm{a}]\,[\mathrm{s}]}+\frac{K_2}{[\mathrm{s}]}+\frac{K_1K_2\,[\mathrm{s}]}{K_3K_5\,[\mathrm{a}]}+\frac{K_1K_2}{K_5\,[\mathrm{a}]}\right)}{\frac{K_1K_2}{[\mathrm{a}]}\left(\frac{1}{K_3}+\frac{1}{[\mathrm{s}]}\right)} \tag{18}$$

$$K_i=\frac{K_{\mathrm{me}}(K_{\mathrm{me}}'+[\mathrm{s}])}{K_{\mathrm{Me}}'}$$

In this formula, K_i represents the *apparent dissociation constant* of each metal ion (Me^{++}) in combination with the enzyme system. Applying this equation to the experimental data, the value of K_i for each inhibitor ion can be calculated, since all the other dissociation constants as well as the concentration of substrate and activator in the experiment are known; thus following values of K_i were obtained for Mn^{++}, Zn^{++}, and Cd^{++}:

$$K_{i\text{-Mn}}=4.2\times10^{-5}\ \text{moles/liter}$$

$$K_{i\text{-Zn}}=1.1\times10^{-6}\ \text{moles/liter}$$

$$K_{i\text{-Cd}}=3.7\times10^{-7}\ \text{moles/liter}$$

The experimental results presented in Fig. 12 will also be accounted for by Equation (17). It is evident from the form of Equation (17) that K_i for each ion is derivable from experimental results shown in Fig. 12, by transforming Equation (17) in terms of $1/V_{\mathrm{me}}$ against $1/[\mathrm{a}]$. The values of K_i in the case of Cu^{++} is computed in this way to be,

$$K_{i\text{-Cu}}=1.4\times10^{-4}\ \text{moles/liter}$$

Inhibition by SH-Poisons—A group of substances known as SH-poisons such as *p*-chloromercuribenzoate and monoiodoacetate were found to inhibit strongly the enzyme reaction. The experimental results are presented in

TABLE II

Effect of Sulfhydryl Inhibitors

Each test solution contained 0.05 *M* glycylglycine, 0.001 *M* $\mathrm{CoCl_2}$, and inhibitor. Experiments were performed at 35° and pH 7.8. The enzyme concentration was 8 μg. protein-N per ml. reaction mixture.

Inhibitor	Concentration	Dgree of inhibition
	(*M*)	(%)
PCMB	10^{-5}	84
"	10^{-6}	57
Iodoacetate	10^{-3}	20

Table II. It remains to be seen whether the SH-group(s) in the enzyme molecule, which is attacked by these substances, plays a role in the combination of the metal ions or of substrate molecule.

SUMMARY

1. Using glycylglycine dipeptidase isolated from baker's yeast, kinetic studies were performed with special reference to the effect of various bivalent ions acting as activators or inhibitors for the enzyme reaction.

2. Among the various metal ions studied, only Co^{++} was found to activate the enzyme reaction over a wide range of concentration.

3. The activity of the enzyme (in the presence of Co^{++} as the activator) is strongly inhibited by Cd^{++}, Cu^{++}, Mn^{++}, and Zn^{++}. The inhibitory action of these ions was found to be competitive with the activating effect of Co^{++}. Under the experimental conditions studied, Ni^{++}, Fe^{++}, and Ba^{++} had no effect on the reaction rate.

4. A scheme of reaction of glycylglycine dipeptidase was proposed, which was shown to be in harmony with the experimental results obtained. It was concluded that a bridge formation by virtue of Co^{++} ion between enzyme and substrate is an essential step in the mechanism of the enzyme reaction.

5. The enzyme was found to be strongly inhibited by SH-inhibitor such as *p*-chloromercuribenzoate and monoiodoacetate.

The author wishes to thank Prof. A. Takamiya and Prof. H. Tamiya for their valuable suggestions and encouragement in this work.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

EFFECT OF HYDROGEN PEROXIDE ON THE LIGHT-INDUCED CAPACITY OF CARBON DIOXIDE-FIXATION IN GREEN ALGAE

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(Received for publication, July 9, 1958)

Previously published experiments (*1–3*) have shown that the photochemical enhancement of CO_2-fixing power of green cells, which was first demonstrated by Calvin and Benson (*4*) in their "pre-illumination" experiment using C^{14} as a tracer, was due to a certain reducing agent(s) which can react with oxidizing agents such as oxygen and quinone. This agent, which was denoted by *R* for the convenience of reference, was assumed to be formed photochemically from its precursor *P* in the following manner:

$$P+H_2O \xrightarrow{\text{light}} R \text{ (or } PH_2)+1/2\ O_2 \qquad (1)$$

It was postulated that the enhancement of CO_2-fixation caused by *R* is a result of the following sequence of reactions (*cf.* Bassham, Benson *et al.*, (*5*)):

$$CO_2+\text{RuDP} \longrightarrow 2\ \text{PGA} \qquad (2)$$

$$2\ \text{PGA}+2\ R \longrightarrow 2\ \text{TP}+2\ P \qquad (3)$$

$$2\ \text{TP} \longrightarrow 6/5\ \text{RuDP} \qquad (4)$$

$$1/5\ \text{RuDP}+1/5\ CO_2 \rightarrow 2/5\ \text{PGA} \qquad (2)$$

and that among these, reaction (2) is the rate-determining step, so that the overall reaction proceeds according to

$$R+3/5\ CO_2 \longrightarrow P+1/5\ \text{PGA} \qquad (5)$$

Our observation that the level of *R* was decreased in the presence of quinone (Q) suggested the occurrence of the reaction

$$R+Q \longrightarrow P+QH_2 \qquad (6)$$

which would entail together with reaction (1) the Hill reaction caused by quinone. Mehler (*6*) showed that the Hill reaction was also brought about by oxygen and that in this process there occurred intermediate formation of H_2O_2 which, under ordinary experimental conditions, was decomposed by catalase. The Mehler reaction was accounted for by the reactions:

$$R+O_2 \longrightarrow P+H_2O_2 \qquad (7)$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O+1/2\ O_2 \qquad (8)$$

of which the latter proceeds faster than the former, so that the overall reaction will assume the form:

$$R+1/2\ O_2 \longrightarrow P+H_2O \qquad (9)$$

It has been observed that when the illuminated cells—in N_2 atmosphere—was suddenly transferred to the darkness, the *R*-level established during the illumination gradually decreased, indicating that the steady *R*-level attained on prolonged illumination was determined by relative rates of photochemical reaction (1) and non-photochemical decay of *R*. This latter reaction was interpreted as being due to the occurrence of reaction (9) caused by the endogenous oxygen that had been formed by reaction (1).

During the course of our studies on the behavior of *R* in *Chlorella* cells we found a peculiar phenomenon regarding the effect of cyanide upon it. It was namely observed that the effect of cyanide appeared entirely analogous with those of oxygen or quinone in that (i) it did not affect reaction (1) and that (ii) it increased the rate of decay of *R* in the dark, and consequently decreased the stationary *R*-level established under continued illumination. Since one could hardly think of an oxidoreductive reaction between *R* and cyanide similar to reaction (6) or (7), we assumed that the main site of attack by cyanide was the catalase in reaction (8) and that the H_2O_2 accumulated thereby would react with *R* in the manner:

$$R+H_2O_2 \longrightarrow P+2\ H_2O \qquad (10)$$

The purpose of this paper is to present data which afford direct evidence for the reaction represented by (10). The concept we have propounded in our previous report that the significance of catalase in the mechanism of photosynthesis lies in the protection of photosynthetic machinery from H_2O_2 has gained further support by this new observation.

MATERIAL AND METHOD

Material used was "dark cells" of *Chlorella ellipsoidea* (*7*), and the experimental procedure was virtually the same as that described in the previous paper (*2*). The algal cells were suspended in 0.02 *M* phosphate buffer (pH 6.8), containing 10^{-4} *M* sodium azide to inhibit catalase activity. This concentration of azide has been shown by Huzisige (*8*) to inhibit completely the catalase activity in intact *Chlorella* cells and to reduce the rate of the Hill reaction (using quinone as oxidant) to 50 per cent of the normal rate. The cells thus pre-treated were subjected to various treatment (illuminated or non-illuminated, in the presence or absence of H_2O_2), and at various intervals aliquots of cell suspension were promptly transferred into dark vessels containing $NaHC^{14}O_3$ (final concentration: 10^{-3} *M*). After being allowed to react with $C^{14}O_2$ for a certain period of time, cells were killed by introducing acetic-hydrochloric acids mixture, and the amount of $C^{14}O_2$ fixed was determined using a Geiger-Müller counter. All the experiments were performed in N_2 atmosphere at 25°; the effective intensity of light applied was about 2 × 10,000 lux.

RESULTS

Effect of Hydrogen Peroxide on the Stationary R-Level in the Light—The cells whose catalase had been completely inhibited by azide were illuminated (in the absence of H_2O_2 and O_2) for 40 minutes when the *R*-level attained a steady value. Then,—under continued illumination—H_2O_2 in varying concentrations were added to the cell suspension, and at intervals the *R*-level was determined by measuring the radioactivity fixed in 60 seconds* of contact of cells with $C^{14}O_2$ in the dark. The results obtained are presented in Fig. 1.

As may be seen from the figure the effect of H_2O_2 was quite similar to that observed with quinone in our previous experiment (*2*). The *R*-level decreased immediately, and in so far as the concentration of H_2O_2 was lower than $10^{-3.3}$ *M*, the *R*-level was sooner or later restored to the original value. When the concentration of H_2O_2 was 10^{-3} *M*, the temporary fall of the curve was more pronounced and the subsequent recovery was only partial. At a still higher concentration of H_2O_2 ($10^{-2.3}$ *M*), the curve fell almost to the zero level and there was no sign of subsequent recovery on continued illumination.

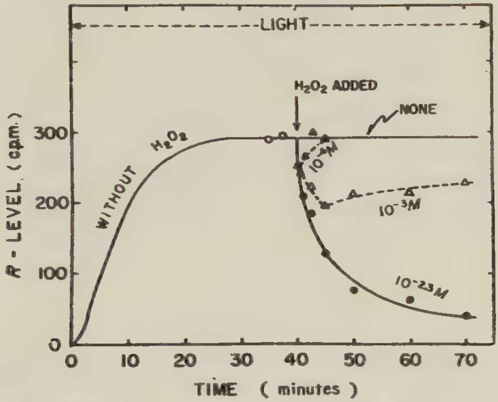

FIG. 1. Effect (in the light of varying concentrations of H_2O_2 on the *R*-level which had previously attained the stationary value in the absence of H_2O_2. Ordinate: c.p.m. of C^{14} fixed in 60 seconds in the dark, Abscissa: the time which the cells spent in the light (prior to the provision of $C^{14}O_2$), first in the absence of H_2O_2 and later in the presence of various concentrations of H_2O_2 as indicated. In this experiment the effect of $10^{-3.3}$ *M* H_2O_2 was also investigated. The result obtained fell between those for 10^{-3} and 10^{-4} *M*.

Effect of Hydrogen Peroxide on the Decay of R in the Dark—The algae, treated with 10^{-4} *M* sodium azide, were illuminated in the absence of H_2O_2 for 40 minutes (with bubbling of N_2), by the end of which a stationary *R*-level was fully established. Then, the light was turned off simultaneously with prompt addition of H_2O_2 to the algal suspension (end concentration: 10^{-3} *M*), and the time course of decay of *R* was followed using $C^{14}O_2$ as a tracer. As the control the decay of *R* in the absence of H_2O_2 was also traced. As may be seen from the results presented in Fig. 2, in which the decay processes are plotted on a semi-logarithmic scale, the rate of decay of *R* in the dark was markedly accelerated by the effect of H_2O_2. The fact that the two curves in the figure started from the same point at zero time indicates, as has been discussed from kinetic point of view in our previous paper (*3*), that the reaction between *R* and CO_2 was not affected by H_2O_2.

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* In our previous paper (*9*), it was shown that sodium azide inhibited reaction (5) and at this concentration it took 60 seconds (instead of 30 seconds in the absence of azide) to complete the reaction.

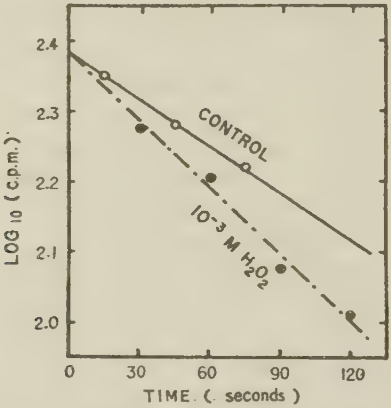

FIG. 2. Semilogarithmic plot of the process of decay of *R* in the dark.

DISCUSSION

The experimental data described above show clearly that H_2O_2, like other oxidizing agent such as quinone and oxygen, reacts with, and consumed by, the reducing agent *R* which is formed in algal cells by the effect of illumination. The fact that, when H_2O_2 was applied in lower concentrations in the light, the *R*-level, after being decreased temporarily, sooner or later restored its original level is obviously due to the exhaustion of H_2O_2 on continued reaction with *R*. Irreversible lowering of the *R*-level observed at higher concentrations of H_2O_2 may be attributed to the destruction by H_2O_2 of some enzyme system involved in the reaction between *R* and $C^{14}O_2$. The experiment reproduced in Fig. 2 showed, however, that in lower concentrations H_2O_2 did not exert any harmful effect upon the reaction between *R* and $C^{14}O_2$. All these findings are analogous to those obtained with quinone in our previous experiment. In view of the analogy between H_2O_2 and quinone in their reactivity with *R*, it may be concluded that H_2O_2—when it exists as such without being decomposed by catalase—functions, equally well as quinone, as an oxidant for the Hill reaction. The results of the present experiment, as well as those reported earlier, indicate that the reactivity of reagents acting upon *R* increases progressively in the order:

$$O_2 < CO_2 < H_2O_2 < \text{quinone}.$$

The available evidence may be taken as a strong support to the view we have developed regarding the mechanism of inhibitory action of cyanide upon photosynthesis and the role played by catalase in the mechanism of photosynthesis. It has been postulated that the main site in the photosynthetic machinery, which is acted upon by cyanide, is the catalase present in chloroplasts. When catalase is blocked by cyanide, H_2O_2 will accumulate as a result of reaction between *R* and O_2 (reaction (7)), and the H_2O_2 thus formed will perform the Hill reaction with *R* (reaction (10)) hindering the reaction between *R* and CO_2 (reaction (5)). The role played by catalase in the mechanism of photosynthesis is to protect the photosynthetic machinery from such hindering action of H_2O_2 which may be liable to be formed in the photosynthetic process occurring in nature.

SUMMARY

1. Using intact cells of *Chlorella*, which had been pretreated with sodium azide (10^{-4} *M*) to inhibit the activity of catalase, investigations were made—by the "pre-illumination" experiments using $C^{14}O_2$ according to Calvin and Benson—on the effect of H_2O_2 upon the photogenic capacity (reducing agent) for CO_2-fixation.

2. It was demonstrated that the level of the $C^{14}O_2$-fixing capacity was markedly lowered in the presence of H_2O_2, indicating that the reducing agent was oxidized by H_2O_2. Based on the fact that the same oxidizing effect has been observed with oxygen and quinone, it was inferred that H_2O_2 shares with oxygen and quinone the property of acting as a Hill oxidant. The reactivity of these oxidants towards the reducing agent in question was found to be in the order:

$$\text{quinone} < H_2O_2 < O_2$$

3. Along the line of reasoning developed in a previous work it was discussed that (i) H_2O_2 is formed in green cells as a result of reaction of O_2 with the reducing agent; that (ii) in normal cells the H_2O_2 thus formed is immediately decomposed by catalase, and that (iii) the inhibitory effect of cyanide upon photosynthesis is due to the inhibition of catalase leading to the accumulation of H_2O_2 which consumes the reducing agent that plays the key role in reducing phosphoglyceric acid to triosephosphate in the mechanism of photosynthesis.

This study was aided by grants from the Nishina Memorial Foundation, the Rockefeller Foundation, and the Ministry of Education. To these bodies we extend our grateful thanks.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

STUDIES ON ARGINASE

II. DISTRIBUTION AND PROPERTIES OF D-ARGINASE (D-ARGININE-SPLITTING ARGINASE)

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(Received for publication, October 23 1957)

Much attention has been given to the possibility that D-arginine will be attacked in the manner comparable with that for the L-isomer, since Felix and Morinaka (*1*) first reported in 1923 that D-arginine was split through the surviving liver. Edlbacher and Zeller (*2*) later demonstrated that arginase of guinea-pig liver gave a feeble action upon D-arginine, but at a high concentration of enzyme D-arginine was split quantitatively into urea and ornithine. Kotake and Nakayama (*3*) and Mabuchi (*4*) observed that extract of intestinal mucosa of the rabbit possesses the higher activity of hydrolysing D-arginine than L-isomer, and they indicated this phenomenon as stereopurification. Sano (*5*) succeeded in separation of the enzyme that is responsible for splitting of D-arginine and designated the enzyme as "hetero-arginase" to distinguish it from L-arginase. According to a recent experiment by Albanese *et al.* (*6*), the human liver extract produces as much urea from DL-arginine as from the L-isomer, while incubation of rat liver extract with DL-arginine yields only half as much urea as with L-arginine. They suggested the possibility that D-ornithine would be produced from D-arginine, provided that the latter is not inverted to L-arginine in the initial stage of the enzymatic processes.

The purpose of the present communication is to describe the distribution in animal organs and several properties of D-arginine-spilitting arginase, which is tentatively designated as D-arginase in the present paper for practical convenience, though, as will be discussed later, no definite evidence has yet been available to belive that the hydrolysis of L- and D-arginine is catalyzed by two different kinds of specific enzymes respectively.

Nevertheless, it has been confirmed that the mouse liver enzyme hydrolyses D-arginine into urea and D-ornithine, the latter compound being isolated from the digest mixture as a dibenzoyl derivative and identified.

MATERIAL AND METHOD

Substrates—D-Arginine hydrochloride was prepared by the enzymatic resolution procedure as described in a previous paper (*7*), and found to be optically pure. DL-Arginine was prepared according to the method of Albanese *et al.* (*6*).

α-*N*-Acyl derivatics of arginine tested were: acetyl-L-arginine (*7*), acetyl-D-arginine (*7*), benzoyl-L-arginine (*8*), and phthalyl-DL-arginine (*9*).

Tissue Homogenate—The animals were killed by decapitation under ether anaesthesia and the liver and kidney isolated immediately after death. One part of fresh tissues was homogenized thoroughly with 10 parts of ice-cold water in a ground glass homogenizer. This homogenate was centrifuged for 10 minutes at 3000 r.p.m. and the supernatant fluid was used as enzyme solution.

Determination of Arginase Activity—The arginase activity was determined either colorimetrically according to the *p*-dimethylaminobenzaldehyde method as described previously (*10*) or by Conway's urease-microdiffusion method (*11*).

In the following tables, the enzymatic activity is expressed in terms of mg. urea found in the incubation mixture after digestion as corrected for appropriate control tests.

RESULTS

Distribution of D*-Arginase in Animal Organs*

The distribution of arginase action upon D-arginine in liver and kidney of various animals was studied and their comparative activities for L- and D-isomers were summarized in Table I. By all kinds of the tissues listed in the table L-arginine was attacked much more readily than the D-isomer. However, under the higher concentrations of enzyme which act on D-isomer and for their a long period of time, *e.g.*, for 24 hours, the activities of these enzyme on the D-one clearly positively were observed. It can be seen from the table that the level of the D-arginase activity is high in the liver of man, rabbit, and mouse; low in the tissues of dog and toad; and only in trace in the tissues of hog, cow, and rat. Albaness *et al.* (*6*) and Reisser (*12*) reported that D-arginine was completely resistant to liver arginase of rat and calf. Table I shows also that D-arginase action of the liver is more powerful than that of the kidney, in general, except for rabbit and toad.

Properties of Mouse Liver D*-Arginase*

Effect of pH—The D-arginase of mouse liver homogenate has a pH optimum of about 9.5 as shown in Fig. 1. The pH-activity relationship was to be essentially the same for both L- and D-arginine used as substrate.

Effect of Temperature—Mouse liver homogenate was heated at 55° in the presence or in the absence of Mn^{++} ion for different periods and its arginase action was assayed by two different methods.

The results are shown in Table II. It has already been pointed out by Hunter (*13*) Rosenthal *et al.* (*14*) that an increase in the L-arginase of activity of homogenates is produced by the addition of Mn^{++} ion and a futher increase occurred when the tissue was incubated at 50°. More recently, Roberts (*15*) reported that preincubation at 50° of dilute mouse liver homogenate (0.2 per cent) in the presence of Mn^{++} ion increased the activity up to about 2.5 time as the activity of the freshly prepared homogenate, whilst preincubation at 50° in the absence of Mn^{++} ion produced no activation. Table II indicates that also at a high concentration of enzyme (1 : 10), the manganese-activation of L-arginase is concident with the finding of Roberts, and that a small decrease in the activity

TABLE I

The Distribution of D-*Arginase in Animal Organs*

The arginase activity, assayed by urease-microdiffusion method, is expressed in mg. urea formed and per cent hydrolysis of arginine added. The reaction mixtures contained 1.0 ml. of 0.014 *M* arginine, 0.5 ml. of enzyme, 0.2 ml. of 0.01 *M* $MnSO_4$. and 0.3 ml. of 0.1 *M* veronal (pH 9.2).

Animal tissues	$MnSO_4$	Arginase activity					
		L-Arginine		D-Arginine			
		1-hour incubation		3-hour incubation		24-hour incubation	
		(mg.)	(%)	(mg.)	(%)	(mg.)	(%)
Cow liver	None	0.880	102	0	0	0.045	5.3
	Added	0.857	100	0	0	0.099	11.5
Cow kidney	None	0.700	81.6	0	0	0.032	3.7
	Added	0.800	93.2	0	0	0.052	6.0
Hog liver	None	0.867	101	0	0	0.088	9.2
	Added	0.800	102	0.010	1.0	0.107	12.4
Hog kidney	None	0.800	93.2	0	0	0.010	1.0
	Added	0.860	100	0.123	14.0	0.299	34.9
Dog liver	None	0.760	88.6	0.111	13.0	0.243	28.3
	Added	0.862	100	0.123	14.3	0.299	34.9
Dog kidney	None	0.630	73.5	0.034	3.9	0.180	24.9
	Added	0.858	100	0.045	5.2	0.225	26.2
Toad liver	None	0.386	45.0	0.010	1.0	0.100	11.6
	Added	0.515	60.0	0.027	3.1	0.148	17.2
Toad kidney	None	0.266	31.0	0.045	5.2	0.211	24.6
	Added	0.328	38.2	0.090	10.5	0.302	35.2
Mouse liver	None	0.857	100	0.133	15.5	0.374	43.5
	Added	0.850	99.0	0.203	23.6	0.432	50.4
Mouse kidney	None	0.810	94.4	0.072	8.4	0.165	19.2
	Added	0.859	100	0.104	12.1	0.198	23.1
Rabbit liver	None	0.883	103	0.090	10.5	0.348	40.6
	Added	0.880	102	0.144	16.7	0.455	54.2
Rabbit kidney	None	0.813	94.8	0.183	21.3	0.546	63.7
	Added	0.875	100	0.218	25.4	0.657	76.6
Rat liver	None	0.684	75.5	0	0	0.045	5.2
	Added	0.858	100	0	0	0.090	10.5
Rat kidney	None	0.270	31.5	0	0	0.019	2.2
	Added	0.443	51.6	0	0	0.029	3.4
Human liver	None	0.703	82.0	0.065	7.5	0.378	44.1
	Added	0.868	101	0.167	19.3	0.500	58.3
Human kidney	None	0.466	54.3	0	0	0	0
	Added	0.660	77.0	0	0	0.018	2.1

is encountered when the homogenate is preincubated at 50° without Mn^{++} ion.

D-arginase, contrarily, has only slightly resistance to heat; about 50 per cent D-arginase activity was destroyed by heating at 55° for 10 minutes in the absence of Mn^{++} ion and there was about 10 per cent decrease in activity even after preincubation with Mn^{++} ion.

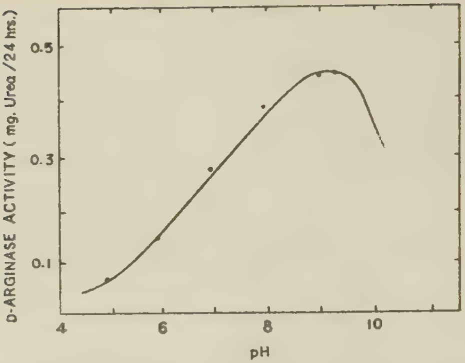

FIG. 1. Relation between pH and mouse liver arginase action upon D-arginine. Assay: urease-microdiffusion method. Incubation for 24 hours. Buffers: CH_3COONa-CH_3COOH for pH 4.0–5.5; K_2HPO_4-KH_2PO_4 for pH 6.0–8.0; NaOH-veronal for pH 8.5–10.0.

TABLE II

Temperature Characteristics of Mouse Liver Arginase

Arginase activity, assayed by two different methods, is expressed in mg. urea formed and per cent hydrolysis of arginine added.

Heating at 55° (*min.*)	$MnSO_4$	Colorimetry				Urease-microdiffusion			
		Agrinase activity							
		L-Arginine[1]		D-Arginine[2]		L-Arginine[2]		D-Arginine[2]	
		3-hour incubation		24-hour incubation		1/3-hour incubation		24-hour incubation	
		(*mg.*)	(*%*)	(*mg.*)	(*%*)	(*mg.*)	(*%*)	(*mg.*)	(*%*)
None (control)	None	2.13	72.0	1.58	53.5	0.711	82.9	0.333	38.8
	Added	2.54	85.7	1.92	64.8	0.867	101	0.405	47.2
10	None	2.08	70.5	0.89	29.9	0.663	77.3	0.131	15.2
	Added	2.96	100	1.61	54.3	0.880	102	0.270	31.5
20	None	2.07	70.0	0.84	28.4	0.657	76.6	0.032	3.7
	Added	3.00	100	1.33	45.0	0.857	100	0.180	21.0

1) 1 : 50 homogenate was used.
2) 1 : 10 homogenate was used.

Effect of Autolysis—After standing at 37° or 0° for different periods of hours, the activity of the enzyme solution for enantiomorphic pair of arginine was determined. The results are summarized in Table III. As shown in the table, arginase for the L-isomer is quite stable in aqueons solution at 0° and shows only little loss of activity even after autolysis at 37°: this fact is consistent with the result of Mohamed and Greenberg (16). On the contrary, arginase for the D-isomer was found to be extermely unstable: the loss of activity was about 20 per cent at 0° and more than 70 per cent at 37° both in 24 hours, and standing for 48 hours at 37° completely abolished the activity. The table shows also that the arginase activity could not be well preserved by the addition of manganous ion to the autolysis mixture.

TABLE III

Stability of Mouse Liver Arginase

The arginase activity, assayed by colorimetry, is expressed in mg. urea formed and per cent hydrolysis of arginine added. The reaction mixtures contained 1.0 ml. of 0.05 *M* arginine, 1.0 ml. of enzyme, 1.0 ml. 0.01 *M* $MnSO_4$, and 1.0 ml. of 0.1 *M* veronal buffer (pH 9.2). The incubation was 1/3 hour for L-arginine and 24 hours for D-arginine, with or without $MnSO_4$.

Autolysis (*hrs.*)	$MnSO_4$	Arginase activity							
		L-Arginine				D-Arginine			
		Temperature of autolysis							
		37°		0°		37°		0°	
		(*mg.*)	(*%*)	(*mg.*)	(*%*)	(*mg.*)	(*%*)	(*mg.*)	(*%*)
0 (Control)	None	2.44	82.7	2.44	82.7	1.58	53.5	1.58	53.5
	Added	2.96	100	2.96	100	1.75	59.2	—	
3	None	—		—		1.26	42.6	1.57	53.0
	Added	—		—		1.56	53.0	—	
24	None	2.38	80.3	2.59	84.0	0.49	16.5	1.13	38.5
	Added	2.62	88.7	2.96	100	0.72	23.1	—	
48	None	2.15	72.6	2.40	81.0	0.03	1.0	1.04	35.5
	Added	2.55	86.1	3.00	101	0.28	9.4	—	

TABLE IV

Per cent Hydrolysis of Arginine and α-Acylarginine by Mouse Liver Arginase

(Assayed by urease-microdiffusion method)

Incubation (*hrs.*)	$MnSO_4$	Arginase activity						
		L-Arginine (*%*)	DL-Arginine (*%*)	D-Arginine (*%*)	Acetyl-L-arginine (*%*)	Acetyl-D-arginine (*%*)	Acetyl-L-arginine (*%*)	Acetyl-DL-arginine (*%*)
20	None	100	45.7	23.6	17.7	2.2	4.7	2.1
	Added	100	54.4	33.1	29.4	8.2	9.4	7.0
40	None	100	53.0	35.7	18.7	8.4	11.5	4.7
	Added	100	62.1	50.6	38.3	11.5	14.2	7.1

Susceptibility of Free and α-N-Acylated L- and D-*Arginine*—As shown in

Table IV, the most susceptible among the substrates tested is L-arginine, and the susceptibility decreases in the following order: DL-arginine D-arginine, acetyl-L-, benzoyl-L-, acetyl-D-, and phthalyl-DL-arginine. The

TABLE V

Effect of Metal Ions on the Action of Mouse Liver Arginase upon D-*Arginine*

(Incubation for 24 hours. Assayed by urease-microdiffusion method)

Addition (0.001 *M*)	Arginase activity (*mg.* urea)	Activity ratio
None	0.360	100
Fe^{++}	0.585	162
Co^{++}	0.558	155
Mn^{++}	0.469	130
Hg^{++}	0	0
Cu^{++}	0.086	24
Al^{+++}	0.302	84
Ag^{+}	0.143	40
Zn^{++}	0.152	42
Li^{+}	0.293	97
Zr^{++++}	0.183	51
Th^{+++}	0.348	97

TABLE VI

Inhibition by Fluoride of the Action of Mouse Liver Arginase upon D-*Arginine*

(Incubation for 24 hours. Assayed by colorimetry)

Addition	Concentration (*M*)	Arginase activity (*mg.* urea)	Inhibition (%)
None	—	0.501	0
NaF	0.02	0.273	45.4
	0.01	0.392	21.8
	0.001	0.485	3.2
NaF.HF	0.02	0.123	75.3
	0.01	0.392	21.8
	0.001	0.444	11.4
Na_2SiF_6	0.02	0.059	88.7
	0.01	0.322	35.7
	0.001	0.404	19.4
CH_2FCOOH	0.02	0.226	54.8
	0.01	0.398	20.6
	0.001	0.475	3.5

last two were almost resistant. It should be pointed out that a marked difference in susceptibilities of L- and D-isomers is almost equally observed both with free and with α-acylated arginine used as substrate.

Effect of Metal Ions—As shown in Table V, the presence of Fe^{++} and Co^{++} produced more than 50 per cent activation of D-arginase and Mn^{++} was somewhat less effective. Almost all the other heavy metal ions tested acted as enzyme inhibitors. The inactivation by Hg^{++} and Cu^{++} was most powerful.

Effect of Fluoride—Table VI shows that 0.02 *M* fluorides exhibited more than 50 per cent inhibition and 0.001 *M* fluorides were less inhibitory. The degree of inhibition decreases according to the following series of compounds: Na_2SiF_6, NaF·HF, NaF, and CH_2FCOOH.

Effect of Ornithine and Lysine—Since ornithine and lysine were reported (*17*, *18*) to be competetive inhibitors of arginase action upon L-arginine, effect of these amino acids upon D-arginine has been tested. The results are summarized in Table VII, which indicates clearly that L-ornithine and L-lysine act as inhibitors also upon D-arginase. L-ornithine and L-lysine caused the strongest inhibition, and D-lysine was less inhibitory than the L-isomer.

Table VII

Inhibition by Amino Acid of the Action of Mouse Liver Arginase upon D-Arginine

(Incubation for 24 hours. Assayed by urease-microdiffusion method)

Addition	Final concentration (*M*)	Arginase activity (*mg.* urea)	Inhibition (%)
L-Ornithine	None	0.292	0
	0.02	0.022	92.3
	0.002	0.151	48.3
	0.001	0.169	41.9
	0.0002	0.191	34.6
L-Lysine	None	0.339	0
	0.002	0.148	56.2
	0.000	0.179	46.8
	0.0002	0.270	20.2
DL-Lysine	None	0.288	0
	0.02	0.135	53.1
	0.002	0.159	44.8
	0.001	0.255	11.5
	0.0002	0.285	1.1
D-Lysine[1]	None	0.304	0
	0.02	0.175	42.2
	0.002	0.249	14.7
	0.001	0.274	8.4
	0.0002	0.303	0

1) A kindly supplied by Dr. T. Murachi (*22*).

Formation of D-Ornithine by the Action of Mouse Liver Arginase upon D-Arginine

A solution of one g. of D-arginine monohydrochloride in 10 ml. of water was mixed 100 ml. of mouse liver homogenate (1 : 10). The mixture was adjusted to pH 9.2 and incubated under toluene at 37° for 48 hours. In order to ascertain the completion of hydrolysis, the pH of the mixture was then ajusted to the optimum pH, 20 ml. of the freshly prepared enzyme solution were added and the digestion was allowed to proceed for another 48 hours. At this time the digest mixture gave only faintly positive Sakaguchi reaction. The mixture was then brought to pH 7.0, and incubated with 5 ml. of 5 per cent urease solution at 37° in order to decompose urea formed by arginase. After digestion for 24 hours the mixture was acidified to pH 4.0, boiled with charocoal for 10 minutes and the coagulated protein was filtered by suction, and washed several times with cold water. The combined filtrate was evaporated *in vacuo* to about 30 ml. and then adjusted with 1 N NaOH to a strongly alkaline reaction to a strongly alkaline reacton to phenolphthalein. After filtration, the product was benzoylated in a usual manner to isolate D-ornithine as its dibenzoyl derivative. The crude material which precipiated on acidification was collected by suction, throughly washed with cold water, air- dried, and repeatedly extracted with small portions of ether and hot petroleum ether. The insoluble residue was dissolved in as small an amount of warm ethanol as possible and acidic water (pH 4) was added dropwise until a faint permanent turbidity occurred. After standing overnight in an ice box, the white precipitate of dibenzoylornithine was collected by suction. The product was recrystallized three times from ethanol-water in the manner described above, air-dried, and shown to be dibenzoyl-D-ornithine by analysis. The yield was 0.3 g.. or 21.4 per cent of the theoretical: m.p. 180° (uncorr.); $[\alpha]_D^{25} = -6.8°$ (c 4, 1 N NaOH). $[\alpha]_D^{20}$ in literature: +7.8 (natural ornithuric acid in NaOH) (*19*); −9.2 (resolution product of D-form, in NaOH, isolated as brucine salt from synthetic dibenzoyl-DL-ornithine) (*20*).

*Analysis** Calcd. for $C_{19}H_{20}H_4N_2$: C, 67.5; H, 5.92; N, 8.23.
Found: C, 66.78; H, 5.86; N. 8.43.

DISCUSSION

The specimen of D-ornithuric acid isolated from the incubation mixture containing D-arginine and the mouse liver homogenate showed somewhat lower m.p. and $[\alpha]_D$ as compared with those reported in literature (*19*, *20*). This may be partly resulted from the long-term incubation of D-arginine in an alkaline medium.

Question then arises whether the enzyme of the mouse liver responsible for splitting of L-arginine is identical with that for breakdown of the D-isomer. Previous papers have described somewhat conflicting data in this respect. Thus, Hunter and Woodward (*21*) pointed out that an unmodified guanido group and a free carboxyl group of arginine molecule are the conditions prerequisite for the action of liver arginase and α-amino group does not offer any point of attachment to the enzyme molecule so that L- and D-arginines are hydrolyzed by the same single enzyme. On the contrary, Sano (*5*) reported a successful separation of two enzymes each responsible for splitting of L- or D-arginine from rabbit intestinal mucosa by means of adsorption on alumina C_γ.

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* Elementary anlysis was performed by the Center of Microanalysis, Kyoto University.

Among the results obtained in the present experiment, the followings may be favorable for the verification of Hunter's hypothesis that one single enzyme, arginase, acts upon both L- and D-arginine; that enzyme actions on L- and D-arginine showed (*1*) similar pH optima (Fig. 1), (*2*) activation by manganous or cobaltous ion in similar way, (*3*) similar patterns of inhibition by lysine or ornithine (Table VII) and by heavy-metal ions (Table V), and (*4*) the activity ratio of arginase actions on L- and D-arginine was almost the same order of magnitude as that of its actions on α-acyl-L- and α-acyl-D-arginines (Table IV).

On the other hand, the observed difference in stability of the enzyme responsible for splitting of L-arginine from that for the D-isomer (Table III), and the different distribution of two enzyme actions among various animal tissues (Table I) may suggest the possible existence of two optically specific enzymes different from one another. However, an attempt to separate these two enzymes contained in mouse liver extract from each other by means of acetone fractionation or adsorption on alumina C_γ at various of pH as reported by Sano (*5*) was unsuccessful, because of instability of the enzyme. Further investigation to solve this kind of complicated problem are now underway.

SUMMARY

The distribution in animal organs and some properties of D-arginase (D-arginine-splitting arginase) have been investigated.

1. The splitting of D-arginine can be quantitatively detected at high enzyme concentrations. The activity is markedly variable depending upon the kind of tissues: it is strong in the liver of man, rabbit, and mouse, weak in tissues of dog and toad, and only in trace in tissues of hog, cow, and rat.

2. D-Arginase is so unstable, in contrast to L-arginase, that the activity is lost by heating at 55°, by autolysis at 37°, and even by standing at 0°.

3. D-Arginase has a pH optimum of 9.5. It is activated by manganous, cobaltous, and ferrrus ions and inhibited by lysine, ornithine, heavy-metal ions, and fluorides.

4. Mouse liver extract has been found to split D-arginine into urea and D-ornithine, the latter compound being isolated from the digest mixture as its dibenzoyl derivative and identified.

The author wishes to express his hearty thanks to Prof. S. Utzino for invaluable suggestions in the course of the present experiment.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

STUDIES ON THE METABOLISM OF LEUCONOSTOC MESENTEROIDES P-60

I. THE REQUIREMENT OF PURINES AS GROWTH FACTORS AND THE EFFECT OF ANTIMETABOLITES ON THE GROWTH

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(Received for publication, April 7, 1958)

It has been demonstrated by several investigators that many microorganisms require purine derivatives for their growth and that their metabolic pathways vary with purine-requirements (*1–5*). *Leuconostoc mesenteroides* P-60 (*Streptococcus equinus* P-60) is reported to require an exogenous purine for its growth. Among the purines, guanine, xanthine or hypoxanthine is required by this organism, but adenine is not essential (*1*, *6*).

Recent advances in the field of purine-antimetabolites have contributed much to the study of bacterial purine metabolism (*7*, *8*), however very little has been published concerning the effects of purine-antimetabolites on this strain. It this article, the authors would like to report some of the observations obtained in this laboratory with respect to the characteristic purine metabolism of *Leuconostoc mesenteroides* P-60.

EXPERIMENTAL

Microorganism—*Leuconostoc mesenteroides* P-60*, which is ordinarily employed for the microbioassay of amino-acids, is the sole strain used in this experiment. The stock culture is maintained in agar medium which contains one per cent each of peptone, yeast-extracts, glucose, sodium acetate and two per cent of agar. The culture is incubated for 24 hours at 37° after inoculation.

Test Medium—The basal medium for test consists of carbon sources, eighteen aminoacids, vitamin-B groups and minerals as shown in (Table I). Test media are prepared by adding to this basal medium purine-derivatives and/or vitamins which would be tested.

Methods—One loopfull of the bacteria from the stock culture is transfered into a tube of liquid medium which consists of the same composition as the stock medium except agar and incubated for 24 hours at 37°. After incubation, the medium is centrifuged and discarded. The precipitate is washed four times with physiological saline solution. The final suspension of the cells is made to give a concentration of 10^{10} cells per ml. Then 0.01 ml. of the suspension is inoculated into each of test-media which contain various compounds studied in this experiment, and incubated at 37°. The test-media are brought to a total volume of 2 ml. in test-tubes, 2 × 10 cm. in size, and sterilized at 100° for 20 minutes before inoculation. The bacterial growth is determined in a spectrophotometer by measuring the optical density of the suspension at 480 mμ.* wavelength after 24 hours incubation unless otherwise noted.

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* This strain was kindly furnished by Dr. K. Kitahara of the Institute of Applied Microbiology, The University of Tokyo.

TABLE I

Basal Medium

Amino acids	(μg./ml.)	Vitamins	(μg./ml.)
DL-Alanine	100	Riboflavin	1
DL-Aspartic acid	200	Thiamine	1
L-Arginine	100	Pyridoxine	1
L-Cystine	50	Pyridoxal	0.2
L-Glutamic acid	250	Ca-pantothenate	1
Glycine	50	Nicotinic acid	1
L-Hystidine	50	Biotin	0.01
DL-Isoleucine	100	Minerals	
L-Leucine	50	K_2HPO_4	500
L-Lysine	100	KH_2PO_4	500
DL-Methionine	100	$MgSO_4 \cdot 7H_2O$	200
DL-Phenylalanine	100	$FeSO_4 \cdot 7H_2O$	0.01
L-Proline	50	$MnSO_4 \cdot 4H_2O$	0.01
DL-Serine	50	NaCl	0.01
DL-Threonine	100	NH_4Cl	1500
DL-Tryptophane	50	Glucose	10 (*mg./ml.*)
L-Tyrosine	50	Na-acetate	5 (*mg./ml.*)
DL-Valine	100	Initial pH	6.8

RESULTS

Effects of Purines, Pyrimidines and Vitamins—The effects of purines, pyrimidines and vitamins in the basal medium on the growth of the bacteria are shown in (Fig. 1). No appreciable growth is observed in non-supplemented basal medium. The presence of para-aminobenzoic acid, pteroylglutamic acid, formyl folic acid, or vitamins (B_{12} *etc.*) shows no growth-promoting-effect. The addition of pyrimidines *per se* has also no stimulatory effects. However, the addition of purine mixtures promotes the growth as well as the complete medium, no further effect is observed by the addition of the vitamins cited above. The addition of a sufficient amount of 4-amino-5-imidazolecarboxamide (AICA), presumably a precursor in purine biosynthesis, has no effect on growth.

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* Quantities of the bacteria are proportional to the optical densities over a wide range around 480 mμ.

Effects of Purines on Growth—Growth effects of many purines are shown in (Fig. 2a). Hypoxanthine, xanthine guanine and isoguanine are effective, but adenine, 2,6-diaminopurine and uric acid are not. Among ribosides of aminopurines, only guanosine shows the effect while adenosine and adenylic acid like adenine are not effective. The response curves of

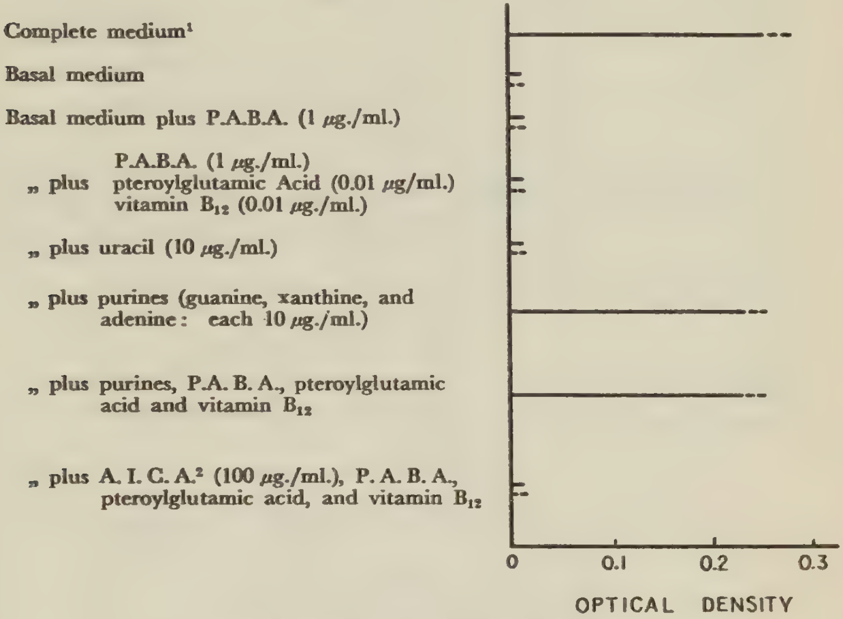

FIG. 1. Effect of purines and/or vitamins.
—— growth in 24 hours.; ---- growth in 3 days

1. Complete medium: Basal medium plus PABA (1 µg./ml.) Pteroylglutamic acid (0.01 µg./ml.), guanine, adenine, xanthine (each 10 µg./ml.) and uracil (10 µg./ml.)

2. AICA: 4-Amino-5-imidazole carboxamide.

hypoxanthine, xanthine, guanine and isoguanine are identical as shown i (Fig. 2b), the maximum growth being at the concentration of *ca.* 40 µg. pe ml. In this case, the addition of pyrimidine (uracil) shows some stimulator effect.

Inhibitory Effects of Several Purine Antagonists—The inhibitory effects of si purine antagonists were tested in the basal medium supplemented wit hypoxanthine (10 µg./ml.). Results are shown in (Fig. 3.). Among thes antagonists, 8-azaguanine has a strong inhibitory action on growth whil 8-azaxanthine shows a weak response. Almost no effects are observed b the addition of 8-azahypoxanthine, 8-azaadenine, 6-mercaptopurine and 2,6-diaminopurine to the media.

Competition between Guanine and 8-Azaguanine—The competitive action o guanine to the inhibitory effect of 8-azaguanine on growth is shown i

Fig. 4a. The inhibitory index (50 per cent inhibition) of 8-azaguanine can be calculated from the response curves of the growth obtained with media containing guanine or guanosine (Fig. 4b). It would be difficult to say that

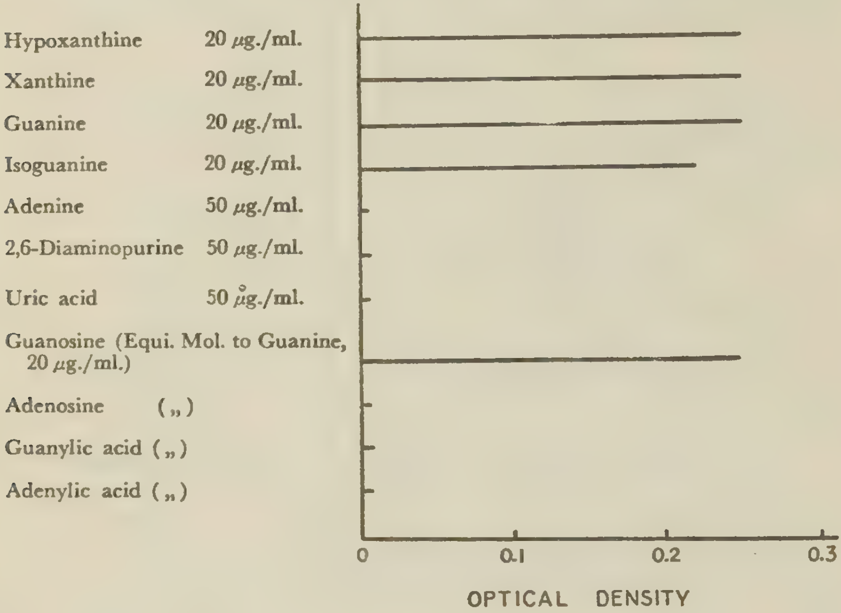

FIG. 2a. Effect of purines.

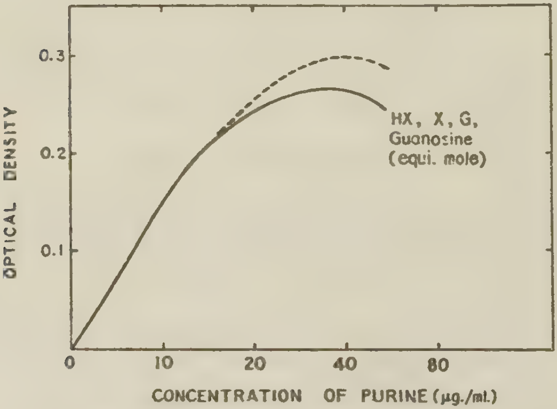

FIG. 2b. Responce curves of purines.
—— Purine without uracil, ---- Purine with uracil (10 μg./ml.)

this might be a competitive antagonism in astrict sense because the inhibition index varied with the concentration of the metabolites in the media, but the inhibition indexes are comparatively constant enough to recognize that

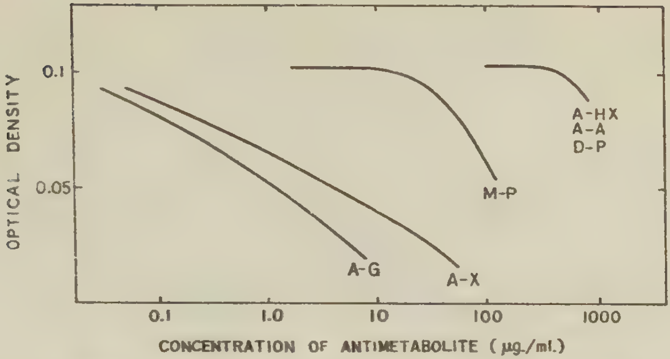

FIG. 3. Growth inhibition action of various purine antimetabolite.
Medium: Basal medium plus hypoxanthine 10 μg./ml. A-G: 8-Azaguanine, A-X: 8-Azaxanthine, A-HX: 8-Azahypoxanthine, A-A: 8-Azaadenine, D-P: 2,6-Diaminocpurine, M-P: 6-Mercaptopurine.

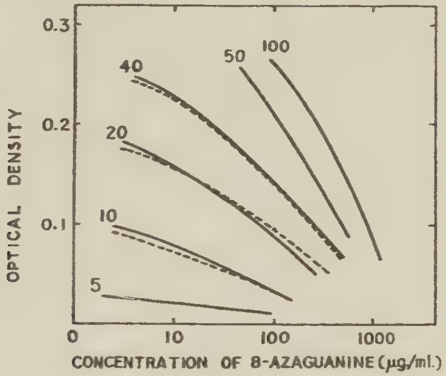

FIG. 4a. Antagonistic action between 8-azaguanine and guanine (guanosine).
Numbers in the graph shows concentration (μg./ml.) of guanine and guanosine (equi. mol. to guanine). ——: Guanine, - - - -: Guanosine.

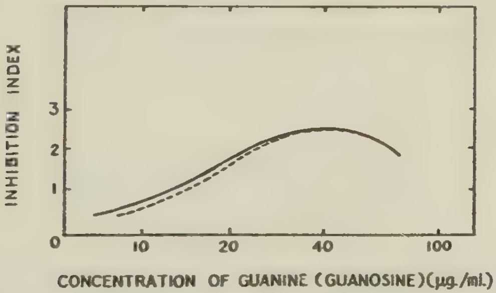

FIG. 4b. Inhibition index of 8-azaguanine.
——: Guanine, - - - -: Guanosine.

$$\text{Inhibition Index} = \frac{\text{Molar concentration of inhibitor}}{\text{Molar concentration of metabolite}}$$

(at 50 per cent inhibition)

there is a remarkable competitive antagonism between 8-azaguanine and guanine (or guanosine) at the optimal concentration of the metabolites.

DISCUSSION

Leuconostoc mesenteroides P-60 repuires preformed exogenous purines as an essential growth factor in the synthetic basal medium (Table I) apparently due to its inability to synthesize some of the purines (Experimental 1, Fig. 1.). Purine derivatives such as hypoxanthine, xanthine, guanine and isoguanine demonstrate a growth-promoting effect, but adenine and 2,6-diaminopurine have no such effect. The inhibitory effect of 2,6-diaminopurine is not pronounced (Exp. 3, Fig. 3). Suzuki (*6*) reported that this strain had an ability to adapt itself to a medium containing adenine, but in this laboratory the adaptation was not distinctly observed while adenine was demonstrated to have a competitive action against 2,6-diaminopurine which prevented the growth at a high concentration*. Hitching *et al.* (*9*) suggested that both adenine and 2,6-diaminopurine shared a common enzyme system in *L. casei*. In this case the fact that both of those purines have no remarkable action on the growth of *L. mesenteroides* P-60 may suggest that the enzyme involving adenine does not exist in this strain, but guanine is required for the system. Among purine antagonists, 8-azaguanine and 8-azaxanthine have remarkable growth inhibiting effect, being observed to be competitive with corresponding purines (Exp. 3,4). Kidder (*10*) observed that 8-azaguanine was competitive with guanosine and guanylic acid in *Tetrahymena geleii* and in the case of guanine its inhibition indexes increased as the amount of added guanine increased. In this strain the response curves of guanine and of guanosine are equimolecularly identical and their inhibition indexes are also similar.

SUMMARY

The purine-requirement and effects of purine antimetobolites in *Leuconostoc mesenteroides* P-60 were studied. This strain required one of the following preformed exogenous purines for growth: guanine, xanthine, hypoxanthine and isoguanine, but not adenine. 8-Azaguanine and 8-azaxanthine showed competitive growth inhibiting effects.

The authors with to acknowledge the helpful advices and suggestions of Dr. Y. Miura and Dr. K. Kitahara of the University of Tokyo.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

X. THE EFFECTS OF VARIOUS AGENTS ON CRYSTALLINE FUMARASE*

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(Received for publication, April 9, 1958)

In the preceding paper (*1*), it was reported that the crystalline fumarase, unlike other crystalline enzymes such as α-amylase, catalase, and alcohol, lactic, and triosephosphate dehydrogenases, is inactivated and digested during the incubation with bacterial proteinase. The present paper deals with additional data on the inactivation of fumarase by bacterial proteinase, and with the study on the effects of various agents on the enzyme protein.

EXPERIMENTAL

Crystalline Fumarase—The enzyme was prepared from pig heart muscle as described in the preceding paper (*1*).

Bacterial Proteinase—Crystalline bacterial proteinase preparation, "Nagarse" was used as described in the preceding paper (*1*), which was supplied by the Nagase Co. Ltd.

Other Proteolytic Enzymes—Crystalline trypsin was kindly furnished by Dr. N. Sakota of Dainihonzoki Co. Ltd. Crystalline carboxypeptidase from beef pancreas was supplied by Professor S. Akabori of Osaka University.

Other Materials—Diphosphopyridine nucleotide was prepared from baker's yeast according to the method of Okunuki *et al.* (*2*). Crystalline adenosine triphosphate was prepared from rabbit muscle by the method of Cohn and Carter (*3*) and Berger (*4*). Ribonucleic acid from brewer's yeast was kindly furnished by Dr. Y. Sugino of Takeda Pharmaceutical Industry Co. Ltd.

Assay of Fumarase Activity—The activity was determined by the spectrophotometric method of Racker as described in the preceding paper (*1*). The values reported here are the initial velocities, and are expressed as decrease in optical density at 300 mμ per unit time under the following conditions: fumarate, 0.0167 M; M/30 phosphate buffer, pH 7.4; final volume, 3.0 ml.; temperature, 30°.

The "per cent inactivation" was calculated from the following equation:

Per cent inactivation=(Initial activity−Residual activity)×100/(Initial activity) (i)

The "per cent residual activity" was calculated from the following equation:

Per cent residual activity=(Residual activity)×100/(Initial activity) (ii)

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* An outline of this work was given at the general meeting of the Japanese Biochemical Society at Fukuoka, in October 1956.

RESULTS

Effect of Proteinase Concentration on Fumarase Activity—In the preceding paper (*1*), it was reported that inactivation of crystalline fumarase by bacterial proteinase is attributable to the proteolytic action of the proteinase. To obtain further information about the interaction between fumarase and proteinase, studies were made as follows.

When fumarase was incubated with various concentrations of bacterial proteinase for one hour, the extent of the inactivation of the enzyme increased with the concentration of the proteinase as shown in Fig. 1. At an equimolar concentration of the two enzymes, fumarase was inactivated to about 50 per cent for one hour. As has been reported previously the molecular weights of fumarase and proteinase are 200,000 (*5*) and 30,000 (*6*), respectively. A 1 : 1 molar ratio corresponds to a 1 : 7 (proteinase : fumarase) ratio on a weight basis. The other enzymes investigated previously (*7*, *8*, *10–13*) were not digested by the proteinase when a 1 : 3–7 ratio on a weight basis was used. Therefore the concentration of proteinase used in present investigation was not very high.

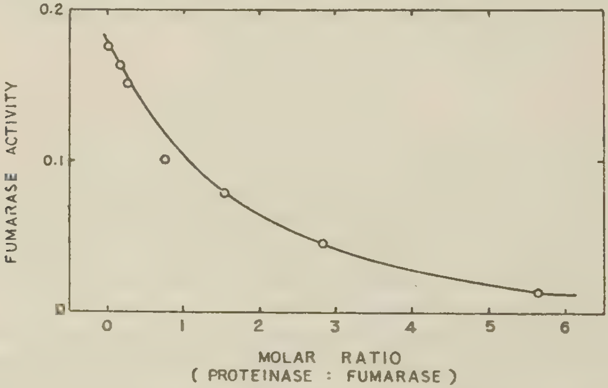

FIG. 1. Relation between concentration of bacterial proteinase and inactivation of fumarase.

1.0 ml. of 0.03 per cent fumarase was incubated with 0.5 ml. of various concentrations (0.035 to 0.00045 per cent) of proteinase for one hour at pH 7.4 and 30°. The final volume of the reaction mixture was 2.0 ml.. 0.5 ml. of the reaction mixture was used to measure activity, as described in the text.

The activity is expressed as the decrease in optical density at 300 mμ in the initial 90 seconds.

When the absolute concentrations of both enzymes were varied keeping their relative concentrations at a molar ratio of 1 : 1, the per cent inactivation of fumarase decreased with its concentrations, as shown in Fig. 2. This effect most probably resulted from a decreased frequency of collision of proteinase and fumarase molecules. In the absence of proteinase, the fumarase activity decreased in proportion to its concentration, and dilution caused no inactivation. It is suggested that fumarase was not changed to a more inactive or digestible form by dilution.

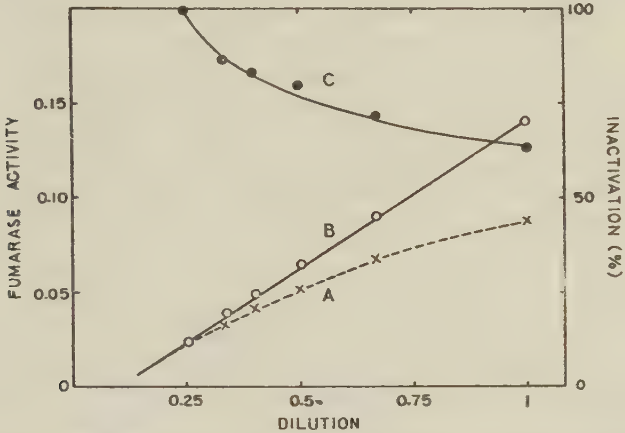

FIG. 2. Relation between fumarase inactivation and concentrations of fumarase and proteinase.

The fumarase concentration varied from 0.0044 to 0.0011 per cent, and bacterial proteinase from 0.00066 to 0.000165 per cent. 1.0 ml. of fumarase was incubated with 1.0 ml. of proteinase, at pH 7.4 and 30°. The molar ratio of the enzymes in the incubation mixture was 1:1 in all experiments.

After incubation, 1.0 ml. of the mixture was used for measuring fumarase activity, as described in the text. The activity is expressed as decrease in optical density at 300 mμ in the initial 90 seconds.

Curve A; Fumarase activity with proteinase. Curve B: without proteinase. Curve C; per cent inactivation.

Effect of Nucleotides and Nucleic Acid on Inactivation of Fumarase by Bacterial Proteinase—Diphosphopyridine nucleotide and adenosine triphosphate have been shown to protect lactic-, alcohol-, and triosephosphate dehydrogenases from denaturation by urea (*7, 8*).

The effect of these nucleotides on the inactivation of fumarase by proteinase was therefore examined. As shown in Table I, they had no protective action. It has been reported that digestion of casein by cathepsin is inhibited by the addition of ribonucleic acid (*9*). However, in the case of inactivation of fumarase by proteinase, yeast ribonucleic acid has no protective action and only a slightly deleterious effect, as shown in Table I.

Effect of Trypsin and Carboxypeptidase on Fumarase—As shown in Fig. 3, trypsin and carboxypeptidase did not inactivate fumarase even at quite high concentrations.

Inactivation of Fumarase from Beef Heart Muscle by Bacterial Proteinase—A crude fumarase preparation from beef heart muscle, like the crystalline enzyme from pig heart muscle, was inactivated by incubation with bacterial proteinase. Therefore inactivation by bacterial proteinase may be a general property of fumarase, at least from animal sources.

TABLE I

Influence of Diphosphopyridine Nucleotide (DPN), Adenosine Triphosphate (ATP), and Yeast Ribonucleic Acid (RNA) on the Inactivation of Fumarase by Bacterial Proteinase

Conditions: Experiment 1; fumarase, 0.014 per cent; proteinase, 0.005 per cent; final volume, 2.0 ml.; incubation period, 60 minutes. Experiment 2; fumarase, 0.012 per cent; proteinase, 0.01 per cent; final volume, 3.0 ml.; incubation period, 30 minutes. Experiment 3; fumarase, 0.018 per cent; proteinase, 0.008 per cent; final volume, 2.0 ml.; incubation period, 30 minutes. Additions were made to the incubation mixture as indicated after being dissolved in buffer and adjusted the pH to 7.4.

1.0 ml. of incubation mixture was used to measure fumarase activity as described in the text.

Experiment number	Additions	Proteinase	Fumarase activity*	Inactivation per cent
1	None	−	0.136	100
	None	+	0.034	25.0
	DPN, 2.5 *mg.*	−	0.130	95.5
	DPN, 2.5 *mg.*	+	0.030	22.0
	DPN, 1.25 *mg.*	−	0.136	100.0
	DPN, 1.25 *mg.*	+	0.035	25.6
	DPN, 0.625 *mg.*	−	0.133	97.5
	DPN, 0.625 *mg.*	+	0.039	28.5
2	None	−	0.116	100
	None	+	0.049	42.2
	ATP, 18 *mg.*	+	0.031	26.7
	ATP, 9 *mg.*	+	0.043	28.5
3	None	−	0.175	100
	None	+	0.090	51.4
	RNA, 3 *mg.*	−	0.085	48.6
	RNA, 3 *mg.*	+	0.065	37.1

* Fumarase activity was expressed as decrease in optical density at 300 mμ during the initial 60 second period.

Inactivation of Fumarase by Urea—Like most other enzymes, fumarase was inactivated by incubation with urea. Fig. 4 shows the inactivation of fumarase by various concentrations of urea. To examine whether the structure of fumarase, which had been partially inactivated by urea, was modified, it was incubated with bacterial proteinase. As shown in Fig. 5, fumarase treated with a low concentration of urea was inactivated more rapidly by proteinase than untreated fumarase. The effect of urea disappeared when it was diluted with buffer solution (curve B). Therefore this rapid inactiva-

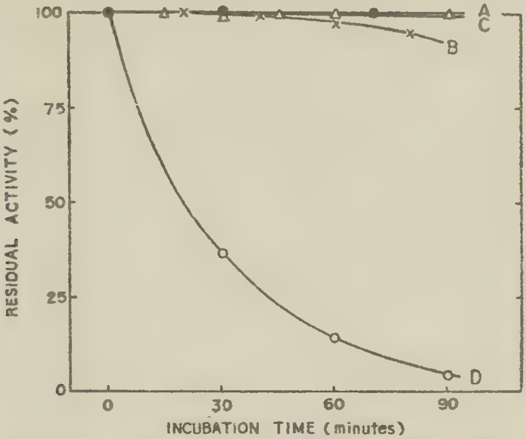

FIG. 3. Action of trypsin and carboxypeptidase on fumarase.

Reaction mixtures; fumarase, 0.015 per cent; trypsin, 0.01 per cent carboxypeptidase, 0.04 per cent; bacterial proteinase, 0.005 per cent. pH 7.4. Temperature, 30°.

Curve A: Fumarase alone. Curve B: Fumarase with trypsin. Curve C: Fumarase with carboxypeptidase. Curve D: Fumarase with bacterial proteinase.

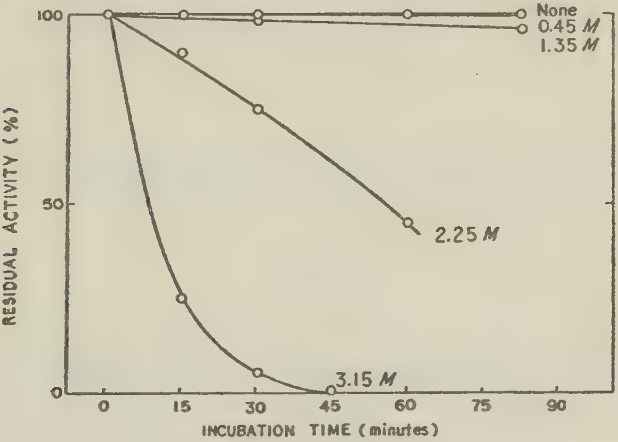

FIG. 4. Inactivation of fumarase by urea.

1.0 ml. of 0.032 per cent fumarase was incubated with 0.5 ml. of urea, in the concentrations indicated, at pH 7.4 and 30°. Final volume, 2.0 ml.

At intervals 0.3 ml. of incubation mixture was used for assay of activity.

tion is probably due to a modification of the structure of the enzyme by urea to a form more easily digestible by proteinase.

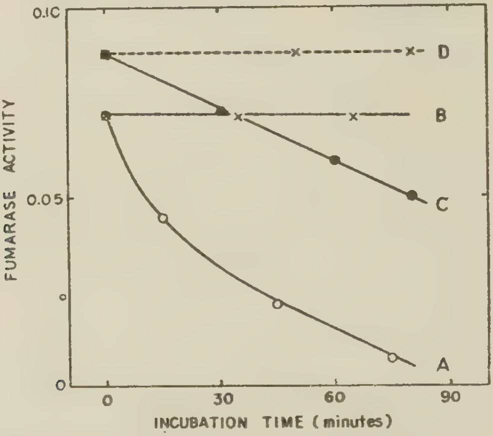

FIG. 5. Inactivation of urea-treated fumarase by bacterial proteinase.

Urea-treatment: 3.0 ml. of 0.03 per cent fumarase was mixed with 3.0 ml. of 4.5 *M* urea and incubated for 30 minutes at 30°.

Incubation with proteinase: Curve A—3.0 ml. of urea-treated fumarase incubated with 0.75 ml. of 0.01 per cent proteinase and 2.25 ml. of *M*/30 phosphate buffer, pH 7.4, at 30°. Curve B—3.0 ml. of urea-treated fumarase incubated with 3.0 ml. of buffer at 30°. Curve C—2.0 ml. of 0.03 per cent fumarase incubated with 1.0 ml. of 0.01 per cent proteinase and 5.0 ml. of buffer. Curve D—2.0 ml. of 0.03 per cent untreated fumarase incubated with 6.0 ml. of buffer.

1.0 ml. of incubation mixture was used for assay of activity as described in the text. Activity is expressed as decrease in optical density at 300 mμ during the initial 60 second period.

DISCUSSION

In the preceding (*1*) and present investigations it was observed that fumarase is easily inactivated and digested by bacterial proteinase without any pretreatment. In previous papers it has been reported that some enzymes, such as amylase (*10–12*), catalase (*13*), and some dehydrogenases (*7*, *8*, *16*), were not destroyed by proteinase even when it was present in a ratio of 1 : 3–7 (proteinase : enzyme, on a weight basis). Fumarase was inactivated to about 50 per cent for one hour at an equimolar concentration of fumarase and proteinase. A 1 : 1 molar ratio corresponds to a 1 : 7 (proteinase : fumarase) ratio on a weight basis. Fumarase was not inactivated by dilution. From these facts, it is considered that inactivation and digestion of fumarase by proteinase is not due to an excessively high concentration of proteinase, or to a dilution of fumarase, and this property of the enzyme is a specific characteristic of it.

Its inactivation by a relatively low concentration of urea seems to suggest that secondary structure of this enzyme is less rigid. However, fumarase in the crystalline state lost little activity during storage for several months in the refrigerator and in solution retained complete activity for several hours at 30°

The oxidized form of crystalline cytochrome c from baker's yeast is digested by bacterial proteinase *(17)*. Glutaminocarboxylase from plants and malic dehydrogenase from pig heart muscle were observed in a preliminary experiment to be rapidly inactivated by bacterial proteinase*. It is, therefore, conceivable that there are two groups of the enzymes, the one resistant to bacterial proteinase and the other digestible even in the active state. The latter group would have a structure comparatively susceptible to bacterial proteinase. The idea that active enzymes have so rigid structures that they are not susceptible to proteinase action without pretreatment *(10–13)*, may therefore be true only in the former group of enzymes.

Some dehydrogenases are converted to holoenzymes in the presence of coenzyme, and these holoenzymes are more stable than their apoenzymes *(7, 8)*. Fumarase does not require the nucleotides as coenzyme. Therefore it is easy to understand that the nucleotides did not protect fumarase from inactivation by proteinase.

Fumarase like other enzymes described in previous reports *(7, 8, 10–13, 16)*, became more susceptible to proteolytic digestion after treatment with urea. It is suggested that the inactivation of fumarase by urea is due to modification of the protein structure to a form more easily digestible by proteinase.

SUMMARY

The relationship between the inactivation of crystalline fumarase and concentration of bacterial proteinase was investigated. Increase in concentration of the proteinase accelerated the rate of the inactivation of fumarase. At a 1 : 1 molar ratio, fumarase was about 50 per cent inactivated in one hour. Fumarase was not inactivated by dilution.

Nucleotides and nucleic acid did not protect fumarase from inactivation by the proteinase.

Like other enzymes, fumarase was inactivated by urea. This inactivation was probably caused by a modification of the structure of the fumarase protein.

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The Journal of Biochemistry, Vol. 45, No. 12, 1958

ADENYLATE KINASE IN HUMAN ERYTHROCYTES

Human erythrocytes contain a highly active adenylate kinase, the activity of which seems to be nearly comparable to that in rabbit muscle. The presence of the enzyme was suggested by the earlier finding obtained in our laboratory that a very rapid equilibration was achieved among the labile phosphate groups of adenosinediphosphate (ADP) and adenosinetriphosphate (ATP) in these cells (*1*). So far much was not known about the human enzyme, though the presence of the enzyme in some animal blood cell and some of its properties were reported by Kotel'nikova (*2*).

Two milliliters of hemolyzate, corresponding to 0.5 ml. of erythrocytes, was incubated with 5 micromoles of ADP at 37°. The paper chromatographic analysis of the reaction products demonstrated a rapid conversion of ADP into adenosinemonophosphate (AMP) and ATP, which was almost complete in less than 20 seconds. Longer incubation resulted in no further appreciable change in the amounts of the three nucleotides, except that AMP was slowly converted into inosinemonophosphate by deamination reaction. These findings were interpreted to indicate that the acivity of the adenylate kinase was much higher than that of the AMP deaminase.

The activity of the enzyme was assayed by coupling the hexokinase with it using terminal phosphate labeled P^{32}-ADP and glucose as substrates. The reaction was allowed to proceed for 5 minutes at 30° and was stopped by addition of diluted sulfuric acid. The solution was kept at 100° for 15 minutes to hydrolyze the remaining ADP. The liberated inorganic orthophosphate was extracted with isobutanol as phosphomolybdic acid on addition of sodium molybdate. The unextracted radioactivity in aqueous layer, which represents the activity of glucose-6-phosphate, was counted. Linear relationship existed between the amount of the adenylate kinase and the rate of conversion of the labile phohphate of ADP to the stable phosphate of glucose-6-phosphate within the range of 0-50 per cent.

The enzyme was somewhat resistant to acid and heat, though the stability is far less than that of the enzyme from rabbit muscle (*3*). This finding conformed with the result of Kotel'nikova. After the treatment of hemolyzate in 0.1 *N* HCl at 60° for 2 minutes nearly 60 per cent of the enzyme activity was recovered. At 90° for 5 minutes the recovery was about 10 per cent. Addition of cysteine to the hemolyzate before or after the heat treatment had no protective effect on the loss in the activity. The heat treatment and the subsequent neutralization resulted in partial purification of the enzyme. A typical result was shown in Table I.

The enzyme activity in hemolyzate was nearly equal between pH 7 and 9 under the condition of the assay method. When the hemolyzate was

TABLE I

Summary of Purification Procedure

One unit of enzyme is defined as that amount which causes the formation of 1 micromole of ATP per minute under the condition of the assay, in which ADP and $MgCl_2$ concentrations are $1\times10^{-3}\,M$ and $3\times10^{-3}\,M$ respectively.

Step	Total protein (*mg.*)	Total units (*μM./min.*)	Specific activity (*μM./min./mg.*)
Hemolyzate (10 ml.) corresponding to 5 ml. of erythrocytes	1550	151	0.097
Filtrate after heating in acid	44	90	2.05

centrifuged, nearly all of the activity was found in the supernatant fraction. Hemolyzate was stored at −15° for months without appreciable loss in the adenylate kinase activity. Erythrocytes from ACD (Acid Citrate Dextrose) blood preserved for six weeks possessed about the same enzyme abtivity with that of fresh cells. The enzyme was also found in sheep and duck erythrocytes, so far as it was examined. In sheep cells the activity was far less than in human cells, reflecting the inert phosphate metabolism recently found in the former cells*.

The presence of such an active enzyme in human erythrocytes, which are metabolically not so active, poses an interesting problem. We have obtained evidence** that the ratio of the quantities of AMP, ADP and ATP in human erythrocytes is dependent of the activity of the enzyme. It seems that this enzyme, if the level of ATP is maintained sufficiently high in the cells, may prevent the accumulation of AMP, which otherwise will irreversibly be deaminated to inosinemonophosphate, thus resulting in lowering of the level of the adenine nucleotides—a serious situation for the viability of the red cells. This problem will be discussed elsewhere with reference to preservation of blood.

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(Received for publication, September 22, 1958)

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*, ** to be published.

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